

ERLING B. MYHRE

**NON-IMMUNE BINDING OF IMMUNOGLOBULIN
G TO GRAM-POSITIVE COCCI**

**DESCRIPTION OF DIFFERENT TYPES OF
IMMUNOGLOBULIN RECEPTORS WITH DEFINED
SPECIFICITIES FOR MAMMALIAN
IgG SUBCLASSES**

Lund 1981

From the Departments of Medical Microbiology and Infectious Diseases
University of Lund, Lund, Sweden

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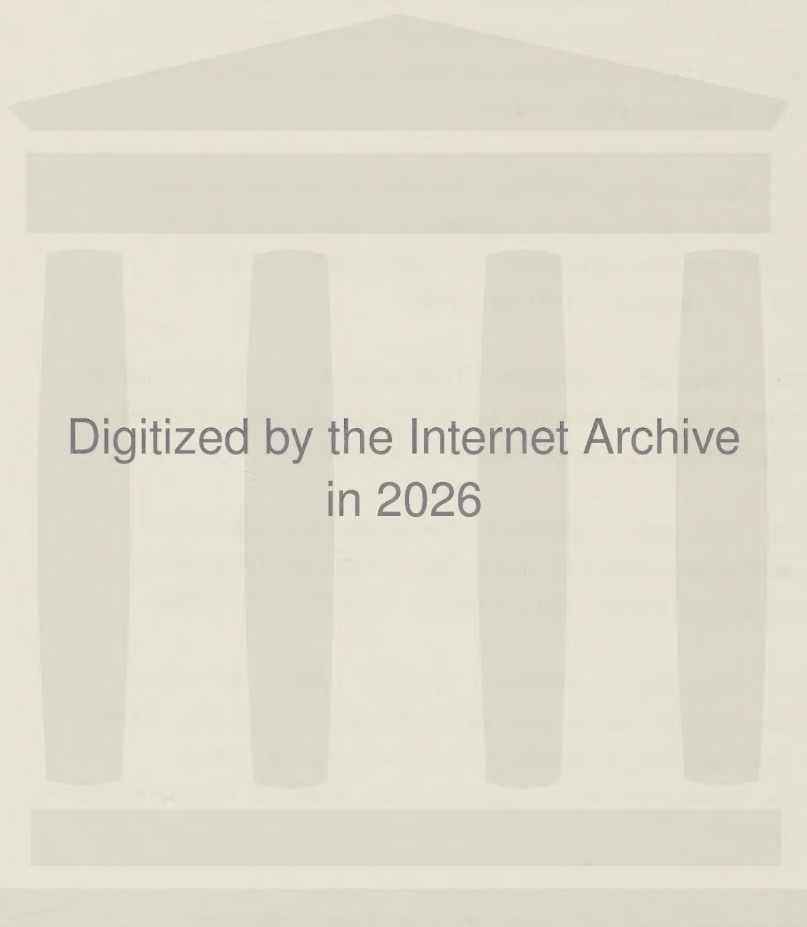


To Alice, Helene and Louise

This thesis is based on the following publications:

- I. MYHRE, E.B. and G. KRONVALL: Heterogeneity of nonimmune immunoglobulin Fc reactivity among Gram-positive cocci: Description of three major types of receptors for human immunoglobulin G. *Infect. Immun.* 17:475-482 (1977).
- II. MYHRE, E.B., O. HOLMBERG and G. KRONVALL: Immunoglobulin-binding structures on bovine group G streptococci different from type III Fc receptors on human group G streptococci. *Infect. Immun.* 23:1-7 (1979).
- III. MYHRE, E.B. and G. KRONVALL: Binding of murine myeloma proteins of different Ig classes and subclasses to Fc reactive surface structures in Gram-positive cocci. *Scand. J. Immunol.* 11:37-46 (1980).
- IV. MYHRE, E.B. and G. KRONVALL: Demonstration of a new type of immunoglobulin G receptor in Streptococcus zooepidemicus strains. *Infect. Immun.* 27:808-816 (1980).
- V. MYHRE, E.B. and G. KRONVALL: Immunochemical aspects of Fc-mediated binding of human IgG subclasses to group A, C and G streptococci. *Mol. Immunol.* 17:1563-1573 (1980).
- VI. MYHRE, E.B. and G. KRONVALL: Specific binding of bovine, ovine, caprine and equine IgG subclasses to defined types of immunoglobulin receptors in Gram-positive cocci. *Comp. Immun. Microbiol. Infect. Dis.* Accepted for publication. (1981).

Individual papers will be referred to by roman numerals.



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CONTENTS

INTRODUCTION

A. Immunoglobulins	9
B. Bacterial cell structure	11
C. Immunoglobulin interactions	12

<u>AIMS OF THE PRESENT INVESTIGATION</u>	14
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EXPERIMENTAL DESIGN

A. Methods for demonstration of non-immune reactivity	15
B. Purification of immunoglobulins	17
C. Iodination of immunoglobulins	19

RESULTS

A. Detection of immunoglobulin reactivity in various bacterial species	21
B. Interaction with non-human immunoglobulins	22
C. Demonstration of different types of IgG receptors	24
D. Specificity of IgG receptor types I-V for mammalian IgG subclasses	27
E. Subclass related differences in receptor affinity	29
F. Binding of isolated IgG fragments to bacteria	31
G. Determination of the IgG binding capacity	32
H. Kinetic aspects of the immunoglobulin binding	32
I. Estimation of the equilibrium constant of the IgG-receptor interaction	33
J. Relationship to other streptococcal reactivities	33

DISCUSSION

A. Definition of different IgG receptor types	34
B. Specificity of defined immunoglobulin receptors	36
C. Immunochemical aspects of the IgG-receptor interaction	38
D. Use of immunoglobulin binding bacteria in immunological work	38
E. Effects on the host-parasite balance	40

<u>GENERAL SUMMARY</u>	41
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<u>ACKNOWLEDGEMENTS</u>	42
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<u>REFERENCES</u>	44
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<u>APPENDIX</u>	55
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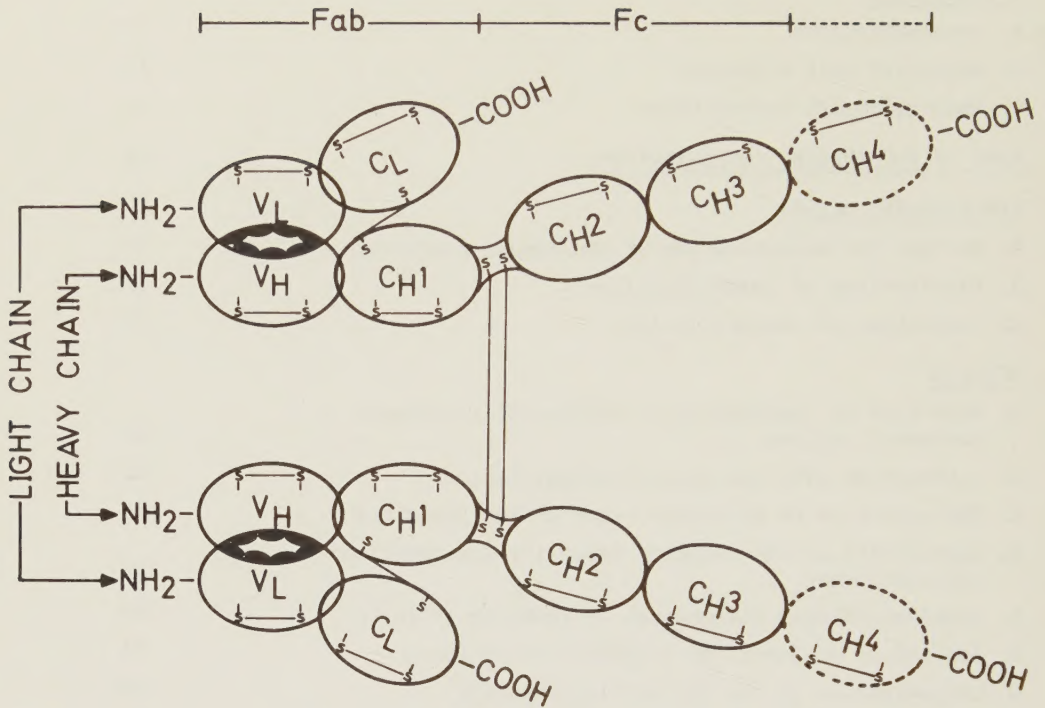


Fig 1. Basic structure of immunoglobulins.

The immunoglobulin molecule is composed of two pairs of light and heavy polypeptide chains linked by disulphide bonds into a Y-shaped structure. Each chain is folded into a series of globular domains. Light and heavy chains consist of two and four domains, respectively. Immunoglobulin M and E contain an additional domain. The hypervariable regions constituting the antigen binding site are shown as dark areas. Digestion with papain cleaves the heavy chains on the amino-terminal side of the inter-heavy chain disulphide bonds resulting in the formation of fragment Fc and two monovalent antigen binding Fab fragments.

INTRODUCTION

A. Immunoglobulins

Immunoglobulins are proteins produced by B-lymphocytes. The immunoglobulin (Ig) molecule consists of one or more unit containing two light and two heavy polypeptide chains, each consisting of approximately 215 and 450 amino acids respectively (12, 22, 29). The four-chain unit is held together by non-covalent forces and by disulphide bridges linking each light chain to a heavy chain and the two heavy chains to each other (Fig 1). Both types of chains are divided into two functionally different regions: an amino terminal V region characterized by variation in the amino acid sequence and a carboxy terminal C region with a rather constant amino acid sequence (21, 22, 96, 147). The V region carries the antibody combining site and is responsible for the recognition and binding of antigen. The constant C region expresses secondary effector functions such as complement fixation, transplacental transfer and interaction with receptors on mammalian cell membranes (101, 147).

Mammalian immunoglobulins can be separated into immunoglobulin classes defined immunochemically by distinct types of heavy chains (39, 101). The immunoglobulin classes differ in their biological properties (130). Many immunoglobulin classes can be further subdivided into subclasses with antigenically cross-reacting heavy chain types (39, 101, 130). There are five human immunoglobulin classes: IgG, IgA, IgM, IgD and IgE defined by heavy chain types gamma, alpha, mu, delta and epsilon, respectively (93, 101, 139). There are two types of human light chains, kappa and lambda, which are common to all IgG classes (129). Immunoglobulin G with an average serum level of 10 to 15 g per liter is the dominating serum immunoglobulin in man (12). IgG occurs as monomeric units with a molecular weight of 150,000 - 160,000 (22). There are four subclasses: IgG1, IgG2, IgG3 and IgG4 constituting approximately 60-75 per cent, 15-30 per cent, 5-10 per cent and 2-7 per cent, respectively, of the total IgG level (40, 101, 132, 138).

The immunoglobulins of higher vertebrate species form a group of closely related proteins with a common basic structure. They have evolved during the phylogeny from a primitive premordial molecule into recognizable immunoglobulin classes and subclasses (39). Immunoglobulin classes analogous to the major human Ig classes have been described in most mammals. Immunoglobulin classes cross-react immunologically with the corresponding classes in related species (103, 105). These cross-reactions reflect the common evolutionary origin, and the degree of cross-reaction correlates with the phylogenetic relatedness of the species (39). IgG subclasses have been described in many mammalian species. Mouse IgG is composed of four subclasses: IgG1, IgG2a, IgG2b and IgG3 (111). The closely related ruminant species cow, sheep and goat, have two subclasses: IgG1 and IgG2 (3, 27, 38, 46, 95). There are four equine IgG subclasses: IgG(a), IgG(b), IgG(c) and IgG(T) (91, 114).

Both the light and the heavy chains of the immunoglobulin molecule can be divided into homology regions, areas of profound structural similarity (21, 26). Each homology region is folded into a compact structure referred to as a domain (Fig 1) (50, 108). All domains are cylindrical in shape and share a basic pattern of polypeptide-chain folding termed the immunoglobulin fold (17). The domain structure consists of straight segments of the polypeptide chain arranged in two layers held together by a disulphide bond. Light chains are composed of one variable (V_L) and one constant domain (C_L) (21, 129). Heavy chains of gamma type contain one variable (V_H) and three constant domains (C_{H1} , C_{H2} and C_{H3}) (21). The constant portion of IgM and IgE contains an additional domain designated C_{H4} (26). The symmetrical Y shape of the immunoglobulin is the result of specific interactions between complementary domains of the two heavy chains.

A number of genetically determined immunoglobulin antigens can be demonstrated (101). Allotypic variability is usually confined to a small area of the Ig molecule and is often limited to a substitution of one or a few amino acids located in the constant portion of the molecule (102). Allotypes are restricted to a single subclass. Several allotypes called Gm types has been identified on human IgG molecules (44, 115). The domains C_{H1} and C_{H2} are linked loosely by an extended segment of the polypeptide chain. This flexible

connection, referred to as the hinge region, is especially susceptible to proteolysis. Digestion with proteolytic enzymes results in defined fragments. Papain splits the IgG molecule into two identical antigen binding Fab fragments and a single Fc fragment consisting of C_{H2} and C_{H3} domains (109, 110).

B. Bacterial cell structure

Bacteria are procaryotic organisms. They have a complex cell wall composed of peptidoglycan (13, 63, 122). Peptidoglycan is a polymer consisting of a backbone with alternating units of N-acetyl-glucose and N-acetyl-muramic acid to which a tetrapeptide is linked. This basic structure is crosslinked into a rigid wall structure. Teichoic acid, another polymeric constituent, forms an integral part of the cell wall of all Gram-positive cocci (61). Most of the teichoic acid is covalently bound to peptidoglycan. A small fraction is, however, associated with the plasma membrane as lipoteichoic acid (144). Peptidoglycan and teichoic acid form a skeleton to which other cell components such as protein and polysaccharide antigens are attached.

Streptococci comprise a large and biologically diverse group of microorganisms characterized by their ability to grow in chains (88). They are widely distributed in nature and can colonize and cause infections in man and in animals (88). Streptococci can be classified in various ways. They elaborate soluble hemolysins and can be classified morphologically as α -, β -, and non-hemolytic strains. A more useful system for classification is the separation into serogroups as described by Lancefield (76, 88). This serological classification is based on the occurrence of distinct carbohydrate antigens which can be extracted from the bacterial cell wall. These group antigens can also be identified by a simple coagglutination method (7). Group A streptococci are responsible not only for acute infections but also for the development of the non-suppurative conditions rheumatic fever and glomerulonephritis (34, 35). This serogroup has therefore been studied in great detail.

Several surface antigens have been demonstrated in group A streptococci (87). The M proteins are the most important of these surface antigens (32). They determine the type specificity of the strains and they constitute an important virulence factor. The virulence of M proteins is expressed by their capacity to inhibit phagocytosis of the organisms by host leucocytes. There are more than 60 different types of group A streptococci, each characterized by a serologically distinct M protein (32). T antigens represent another series of surface antigens present in most group A strains (87). A large number of T antigens have been defined. This antigen system is primarily used for typing of clinical isolates (87). R antigens represent a third class of surface proteins (88). The topographical arrangement of the surface antigens and their relation to other cell wall components is not known. Electronmicroscopic studies of group A streptococci have revealed a rough surface structure with long filamentous appendages (135). Streptococcal species other than group A have received less attention. M proteins have been demonstrated only in a few group G streptococcal strains (86). It is, however, reasonable to think that group C and G streptococci which in other respects resemble group A strains, also have a complex surface structure with distinct antigens.

C. Immunoglobulin interactions

Immunoglobulins are essential elements in the host defence against invading microorganisms. This central role in the host-parasite balance is reflected in the organization of the antibody molecule into two functionally different regions (108, 147). Antibodies can recognize different antigens with great specificity. Binding of an antigen to the variable Fab region initiates secondary effector functions designed to eliminate or immobilize the antigen (147).

This regular immune reactivity can be "shortcircuited" when proteins interact directly with immunoglobulin structures other than the antigen binding site (73). The reactive structures are usually found in the Fc portion of the molecule. These interactions are not related to normal antibody activity and they are referred to as non-immune immunoglobulin reactions. Fc reactivity is a more specific term for interactions involving the Fc portion of the immunoglobulin molecule.

More than 30 years ago Jensen showed that staphylococci contained an extractable product, antigen A, which precipitated with a collection of normal human sera (53). Jensen interpreted the precipitation reaction as a true antigen-antibody reaction due to normally occurring antibodies to staphylococci. Further studies by Löfkvist and Sjöquist revealed that the original antigen A of Jensen was a protein constituent of the bacterial cell wall (83). They adopted the name, protein A, which was proposed by Grov et al. (43). The non-immune nature of the protein A reactivity was discovered by Forsgren and Sjöquist (31). They studied the reactivity of proteolytic IgG fragments and found that the protein A reactivity was associated with the Fc fragment. Kronvall and co-workers showed that protein A reactivity could be demonstrated in nearly all mammalian species (70). They also showed that closely related immunoglobulins such as IgG subclasses can differ in protein A reactivity. Human IgG1, IgG2 and IgG4 were found to be reactive in contrast to the IgG3 subclass which was completely protein A negative (74). Similar observations have later been made in mammalian species including the mouse (68), the rat (92) and the cow (81).

Recent studies have revealed that protein A expresses two different non-immune reactivities: a classical Fc mediated reactivity (31, 66) and a newly detected reactivity involving structures on the Fab portion of the immunoglobulin molecule. Fab mediated protein A reactivity was observed in experiments performed by Grov and Endresen in 1976 (42) but the nature of the interaction was first recognized by Endresen in 1979 (24). Further evidence for Fab mediated protein A reactivity was obtained from studies of the interaction between protein A and human polyclonal IgE (55). Inganäs and Johansson found that the protein A reactivity of IgE resided in the Fab region (51). Protein A reactivity involving Fab structures has also been demonstrated in non-human immunoglobulin classes (97, 149, 150).

A streptococcal non-immune reactivity analogous to the staphylococcal protein A reactivity was described in 1973 by Kronvall (64). He found that human IgG myeloma proteins representing all four subclasses interacted with a surface structure present on β -hemolytic group A, C and G streptococci. This non-immune binding was further investigated by Christensen and co-workers (6-9). They observed that group A streptococci interacted with human IgA, a reactivity which appeared to be unrelated to the IgG reactivity of this serogroup (10).

AIMS OF THE PRESENT INVESTIGATION

The main objective of the present studies was to perform a basic systematic investigation of the non-immune binding of mammalian immunoglobulins to bacterial cells. Experiments were designed to explore the following aspects of the bacterial - immunoglobulin interaction:

- Distribution of immunoglobulin reactive structures in various bacterial species
- Identification of different types of immunoglobulin binding surface structures (immunoglobulin receptors)
- Determination of the specificity of defined types of IgG receptors for mammalian immunoglobulins including IgG subclass immunoglobulins
- Exploration of the kinetics of the receptor-IgG interaction including determination of the association and dissociation parameters, and estimation of the equilibrium constant of the binding
- Delineation of the relationship between the immunoglobulin reactivity and the capacity to bind other mammalian proteins

These studies were carried out with two main perspectives in mind:

1. Non-immune binding of immunoglobulin might shortcircuit the regular antibody reactivity and may be of significance in the host-parasite relation.
2. Immunoglobulin reactive bacteria bind antibodies irrespective of their antigen specificity and can therefore be used as laboratory reagent for selective absorption of immunoglobulins

EXPERIMENTAL DESIGN

A. Methods for demonstration on non-immune reactivity

Non-immune interactions between immunoglobulins and microorganisms can be studied by various techniques. The staphylococcal protein A reactivity was originally investigated in a gel precipitation system (74). This method can only be used for studies employing soluble immunoglobulin binding components. Another limitation of this assay system is the failure of certain immunoglobulins such as human IgM and IgA, to form detectable precipitates. Demonstration of protein A reactivity in these two immunoglobulin classes requires direct binding methods (45).

Direct binding assays employing labelled immunoglobulins constitute a versatile and powerful tool for studies of non-immune reactions. The sensitivity of the test system is determined by the ratio between the immunoglobulin quantity and the number of bacteria used in the experiment. High sensitivity is accomplished when a small amount of immunoglobulin is tested for binding to a large number of bacteria. In contrast, experiments performed with a relative excess of immunoglobulin molecules will reflect quantitative aspects of the immunoglobulin reactivity. Such an experimental setting is used to determine the maximal binding capacity of bacteria.

A crucial point in the binding assay is the quality of the immunoglobulin preparations. All immunoglobulin fractions must have a high degree of purity. It is therefore necessary to determine and to report the purity of the preparations. Immuno-electrophoresis can be used for this purpose. But to ensure that the radiolabelled protein really represents the immunoglobulin to be studied additional tests should be performed. For this purpose iodinated immunoglobulin preparations are mixed with normal serum and a regular immuno-electrophoresis is run. The slides are preserved and then analyzed by autoradiography. By use of appropriate antisera it can be shown whether the label is associated exclusively with the protein to be studied or not. An alternative approach is

to do quantitative precipitation studies. When precipitation is carried out at equivalence between antigen and antibodies the purity can be expressed quantitatively as per cent precipitability (78).

Another critical point in binding assays is the non-specific absorption of proteins to test tubes and to bacteria. This unspecific binding is related to the protein concentration. The lower the protein concentration the more pronounced is this undesired binding. The problem can, however, easily be overcome by addition of other proteins such as human serum albumin. An alternative to the employment of other proteins to cover unspecific binding sites, is the use of non-ionic detergents. This group of agents are very efficient and can almost completely eliminate the non-specific uptake. In the present investigation Tween 20 was incorporated in all buffers at a concentration of 0.05 to 0.1 per cent. This procedure had no effect on the specific binding of immunoglobulins to bacterial surface structures.

Addition of increasing amounts of unlabelled immunoglobulins results in a dose-dependent inhibition of the uptake of radiolabelled immunoglobulin. Such inhibition experiments can be used for studies on unfractionated immunoglobulin preparations including serum samples. The competitive nature makes it possible to compare the binding properties of different immunoglobulin preparations. This experimental design can also be used to explore the spatial relationship between different types of binding sites on the bacterial cell surface. A capacity of one protein to inhibit the binding of another protein suggests that these two proteins interact either with the same structure or with two adjacent structures.

Non-immune interactions can also be studied by hemagglutination procedures. Sheep red blood cells coated with rabbit anti-sheep erythrocyte antibodies have been used for detection of immunoglobulin binding potentials (8, 126, 146). A more sensitive and versatile test system employs human red blood cells coated with anti-Rhesus antibodies (5, 120). The hemagglutination procedures require soluble immunoglobulin reactive components extracted from cultured bacteria and they cannot be used for experiments involving intact organisms.

B. Purification of immunoglobulins

Direct binding experiments were performed with purified immunoglobulin preparations isolated from serum samples from six mammalian species belonging to four orders (Table 1). Three biochemical purification methods were used: preparative electrophoresis (PE), ion exchange chromatography (IEC), and gel filtration (GF). A combination of these methods was often used to achieve a high degree of purity (Table 1). Human and murine immunoglobulins were monoclonal proteins isolated from myeloma sera.

Preparative electrophoresis: Zone electrophoresis was performed in a 0.6 per cent agarose gel using 0.0075 M Veronal buffer pH 8.6 (54). The gel was cut into slices and the protein eluted from the slices with phosphate buffered saline.

Ion exchange chromatography: Ovine, caprine and equine serum was obtained from non-immunized animals. IgG subclass immunoglobulins were isolated from serum samples using preswollen DEAE-Sephacryl. Ovine IgG2, caprine IgG2 and equine IgG(ab) were eluted with 0.01 M sodium phosphate buffer pH 0.8. Ovine IgG1, caprine IgG1 and equine IgG(c) and IgG(T) were eluted with a linear NaCl gradient.

Gel filtration: Gel filtration on a Sephadex G-200 column was often used as a final step to remove contaminating proteins and aggregated immunoglobulins.

Purified immunoglobulin fractions were concentrated either in collodion sacks or by ultrafiltration (Amicon, Diaflow ultrafilter PM 30). All preparations were analyzed by gel precipitation or immunoelectrophoresis using appropriate antisera.

Table 1. Mammalian immunoglobulin G subclasses tested for binding to Gram-positive cocci.

Order	Species	Immuno-globulin	No of isolated myeloma proteins	Purification methods*
Primates	Man	Human IgG1	6	PE, GF
		Human IgG2	4	PE, GF
		Human IgG3	6	PE, GF
		Human IgG4	3	PE, GF
Rodentia	Mouse	Murine IgG1	1	IEC**
		Murine IgG2a	2	IEC**
		Murine IgG2b	2	IEC**
		Murine IgG3	2	IEC**
Artiodactyla	Cow	Bovine IgG1		IEC**
		Bovine IgG2		IEC**
	Sheep	Ovine IgG1		IEC
		Ovine IgG2		IEC, GF
	Goat	Caprine IgG1		IEC,
		Caprine IgG2		IEC, GF
Perissodactyla	Horse	Equine IgG(ab)		IEC, GF, PE
		Equine IgG(c)		IEC, GF, PE
		Equine IgG(T)		IEC, GF, PE

* PE, GF and IEC denote preparative electrophoresis, gel filtration (Sephadex G-200), and ion exchange chromatography, respectively.

** Commercially available preparations.

C. Iodination of immunoglobulins

The present studies were performed with immunoglobulin preparations labelled with the gamma-emitting isotope I^{125} . Three different iodination methods were used: chloramine-T (89), mild electrolysis (45, 116) and solid-state lactoperoxidase (16). The basis for all three methods is the provision of an oxidative environment which results in incorporation of isotope atoms into the protein molecule. Introduction of iodine atoms can alter the properties of the molecule (104). This risk is relatively high for low molecular weight compounds but is of less significance for high molecular weight proteins such as immunoglobulins. The real risk for changes in the physiochemical properties lies in the exposure to the oxidative environment during the labelling step (104). It is therefore necessary to keep the oxidative potential at such a low level that efficient iodination is accomplished but without adverse effects on the protein structure. The three procedures used in the present investigations are mild, and they do not alter the native protein. Use of high concentration of chloramine-T results in a reduced immunoglobulin reactivity as revealed by low levels of IgG uptake.

Altered binding properties due to iodination can be assessed in binding assays employing defined mixtures of labelled and native protein. Such experiments showed that immunoglobulins labelled by the solid-phase lactoperoxidase procedure did not differ from the native preparation in terms of uptake levels (Paper V). Lactoperoxidase labelled immunoglobulin preparations were used to determine the parameters of the receptor-IgG interaction.

Table 2. Distribution of non-immune immunoglobulin G reactivity among Gram-positive bacterial species.

Bacterial species	No of strains	Immunoglobulin reactivity
Group A streptococci	30	+++
Group B streptococci	40	-
Group C streptococci		
<u>Streptococcus equisimilis</u>	30	+++
<u>Streptococcus zooepidemicus</u>	18	+++
<u>Streptococcus equii</u>	7	+
<u>Streptococcus dysgalactiae</u>	12	+++
Group D streptococci	30	-
Group G streptococci		
β -hemolytic human strains	30	+++
α -hemolytic bovine strains	18	-
β -hemolytic bovine strains	16	+
Group M streptococci	6	-
Group N streptococci	11	-
Group P streptococci	10	-
Group U streptococci	14	-
<u>Streptococcus uberis</u>	2	-
<u>Staphylococcus aureus</u>	40	+++
<u>Staphylococcus epidermidis</u>	10	-
<u>Staphylococcus saprophyticus</u>	10	-
<u>Diplococcus pneumoniae</u>	40	-

Positive signs denote low (+) and high (+++) degree of binding of human IgG, respectively.

RESULTS

A. Detection of immunoglobulin reactivity in various bacterial species

(Papers I, II, IV and V).

Gram-positive cocci representing 19 species were examined with a sensitive binding assay for non-immune binding of human immunoglobulin G (Paper I). Bacteria were cultured overnight in liquid culture medium, washed free from broth and suspended in phosphate buffered saline pH 7.2. The experiments were performed with radiolabelled immunoglobulin and with a relative excess of intact bacterial cells. Five to ten per cent of the labelled ligand was trapped non-specifically in the bacterial pellet. Positive binding was defined as an immunoglobulin uptake exceeding the binding noted with non-reactive control strains. The degree of immunoglobulin reactivity was expressed as percentage of radioactivity bound to the bacterial pellet. The results of direct binding assays were highly reproducible. In one series of experiments uptake levels could be measured with an impression of 5.5 per cent (coefficient of variation) (Paper IV).

Definite binding of human immunoglobulin G was noted in Staphylococcus aureus strains and in group A, C and G streptococci (Fig 1 in paper I and Fig 1 in paper II). These results are summarized in Table 2. Seventy human group B and D streptococcal strains were completely non-reactive. The group D streptococci represented the species Streptococcus faecium, Streptococcus durans and Streptococcus faecalis. Forty pneumococci isolated from clinical specimens were incapable of binding polyclonal human IgG. Several other bacterial species including Staphylococcus epidermidis, Staphylococcus saprophyticus, Streptococcus uberis, β -hemolytic group M, N, P and U streptococci have recently been tested for non-immune immunoglobulin reactivity but with negative results (Table 2, unpublished results).

All Staphylococcus aureus strains were homogeneous in their reactivity, with binding of 60-75 per cent of added polyclonal human IgG.

Group A streptococci formed a heterogeneous group. The majority of the strains showed a positive binding. The degree of reactivity was, however, quite variable with uptake levels of 10-35 per cent. Approximately 30 per cent of group A strains isolated from consecutive clinical specimens were non-reactive.

Group C streptococci can be classified into four different species: Streptococcus equisimilis, Streptococcus dysgalactiae, Streptococcus zooepidemicus and Streptococcus equi (18). Studies of strains representing these species showed that all four species were immunoglobulin reactive (Table 1 in paper IV). Most of the strains tested were positive but there was great variability between individual strains with uptake levels ranging from 20 to 80 per cent. Streptococcus equi strains were less reactive than other group C species.

Group G streptococci can cause infections in both human and animal hosts. The immunoglobulin reactivity of three different series of group G strains were studied: β -hemolytic human strains, β -hemolytic bovine strains and α -hemolytic bovine strains (paper II). The α - and β -hemolytic bovine isolates had been recovered from milk samples obtained from cows suffering from mastitis. Human β -hemolytic strains were rather homogeneous. Almost all human strains showed a high degree of reactivity with uptake levels of 60-90 per cent (Fig 1 in paper II). Beta-hemolytic bovine strains, morphologically identical with the human strains formed two groups; one group with completely negative strains and another consisting of strains demonstrating a low but definite binding of IgG. None of the examined α -hemolytic bovine isolates showed any reactivity.

B. Interaction with non-human immunoglobulins

(Papers I and VI).

Bacteria capable of binding human IgG can also interact with other mammalian immunoglobulins. No strains has yet been found which can bind non-human immuno-

globulins only. The reactivity of animal immunoglobulins was assessed in inhibition experiments using a panel of sera collected from unimmunized animals. In a pilot study increasing amounts of normal human serum were mixed with a constant amount of radiolabelled human IgG and the uptake of labelled immunoglobulin to a defined number of bacteria determined. The amount of human serum which was required for complete inhibition was noted (Fig 2 in paper I). Equal quantities of animal sera were then tested in identical experiments. The degree of inhibition noted in such an assay system is determined by the serum concentration of the various immunoglobulin classes and subclasses and their affinity for the binding sites on the bacterial cell surface. Thus the observed inhibition is therefore the result of interactions involving more than one type of immunoglobulin molecules and it reflects the reactivity of the total immunoglobulin fraction in the sample. Despite the complexity of the interaction three different inhibition profiles were found (Fig 3 and table 1 in paper I). These profiles could be correlated to defined bacterial species.

A characteristic inhibition profile was obtained with all Staphylococcus aureus strains. Rabbit, guinea pig, dog, cat and pig serum inhibited efficiently the uptake of iodinated human IgG to staphylococci. This observation suggests that immunoglobulin from these species expresses a high affinity for protein A. In contrast, the lower inhibiting capacity of rat, horse, sheep and cow serum indicate that the immunoglobulin fractions on a whole has a lower degree of protein A reactivity. The results of direct binding experiments employing purified IgG subclass immunoglobulins are compatible with this observation (Table 1 in paper VI). A definite binding to staphylococci was noted with bovine IgG2 and ovine IgG2 but not with the IgG1 subclass in the two species. However, a weak protein A reactivity was found when bovine IgG1 and ovine IgG1 were tested for binding to a protein A Sepharose matrix.

Group A streptococci presented an inhibition profile that was clearly different from the pattern observed with staphylococci. Significant inhibition was seen only after addition of rabbit and pig serum. All other sera had only minimal inhibiting capacity. These results suggest that group A streptococci which can bind human IgG are restricted in their specificity for non-human immunoglobulins. Direct binding experiments performed with purified immunoglobulin preparations showed that group A streptococci do not bind murine, bovine, caprine and

equine IgG (Table II in paper III and Table 1 in paper VI). Recent studies have, however, revealed that polyclonal rabbit IgG can interact with type II receptors with binding levels of approximately 80 per cent (Unpublished observation).

Human group C and G streptococci formed a homogeneous group with a common inhibition profile, Rabbit, guinea pig, horse, pig, sheep and cow serum had a high inhibition capacity. Rat, dog and cat serum was less inhibitory. This observation indicates that group C and G strains have a broad reactivity interacting with most mammalian immunoglobulins. Studies performed with purified immunoglobulin from several species confirmed that these two streptococcal species have a broad uniform reactivity (Table 1 in paper VI).

C. Demonstration of different types of IgG receptors

(Papers II, IV, V and VI).

Inhibition experiments performed with a panel of mammalian sera revealed three different inhibition profiles. Strains representing individual bacterial species presented inhibition profiles of profound similarity. This observation suggested that there were three different types of binding sites for human immunoglobulin G, each confined to defined bacterial species. Direct binding studies employing isolated immunoglobulin fractions confirmed the existence of three major types of binding sites (Table 2 in paper V and Table 1 in paper VI). These binding sites were in the following studies referred to as IgG receptors type I-III. The first described immunoglobulin binding component in bacteria, staphylococcal protein A, represents type I receptors. Type II receptors are found only in group A streptococci. Type III receptors are carried by β -hemolytic human group C and G streptococci. Type III receptors were in subsequent studies also demonstrated in hemolytic bovine group C streptococci belonging to the species Streptococcus dysgalactiae.

Systematic exploration of other bacterial species with purified mammalian immunoglobulin fractions resulted in the detection of two additional IgG receptor types: type IV found on bovine β -hemolytic bovine group G strains

Table 3. Distribution of different types of immunoglobulin G receptors in defined bacterial species.*

Bacterial species	IgG receptor type
<u>Staphylococcus aureus</u>	I**
Group A streptococci	II
<u>Streptococcus equisimilis</u>	III
<u>Streptococcus dysgalactiae</u>	III
Human group G streptococci	III
Bovine β -hemolytic group G streptococci	IV
<u>Streptococcus zooepidemicus</u>	V

* Receptor types are defined by their specificities for mammalian IgG subclasses

** Type I receptors represent protein A

(Paper II) and type V confined to the group C species Streptococcus zooepidemicus (Paper IV).

The structure of protein A (receptor type I) has been studied in great detail and its amino acid sequence has also been determined (49, 124, 125). The other IgG receptor types are so far defined by their specificity for mammalian IgG subclasses. The distribution of receptor types I-V in various bacterial species is summarized in Table 3.

The sensitivity of the immunoglobulin receptors to proteolytic enzymes was studied by digesting bacterial suspension with increasing amounts of enzyme for defined periods of time (Paper IV). The proteolytic process was stopped, the bacteria washed and tested for IgG uptake. Selected bacterial strains carrying type III and type V receptors showed marked reduction in uptake levels after exposure to pepsin and trypsin. Thus the IgG receptors in streptococci are sensitive to both enzymes. Receptor type V of Streptococcus zooepidemicus was found to be extremely sensitive to trypsin in contrast to the more stable type III receptors found on Streptococcus equisimilis and Streptococcus dysgalactiae. Pepsin digestion had a slightly different effect. Type V receptors were very pepsin sensitive. Type III receptors carried by Streptococcus equisimilis and Streptococcus dysgalactiae strains differed in pepsin sensitivity. The receptors carried by the former species were more resistant than the corresponding receptors present on Streptococcus dysgalactiae strains.

IgG receptor types I-V are all heat stable. Bacteria which had been heated to 80°C for 5 minutes did not differ in immunoglobulin reactivity from fresh untreated organism. Bacterial cells conserved by heat treatment could be stored as a suspension at 4°C for prolonged periods of time without loss of reactivity (Paper V).

Immunoglobulin reactive strains maintained in the laboratory by serial passages on blood agar plates for more than one year were still capable of binding IgG (Paper IV).

D. Specificity of IgG receptor types I-V for mammalian IgG subclasses

(Papers III, IV, V and VI).

Binding experiments were performed with isolated IgG subclass immunoglobulins from six mammalian species belonging to four different orders (Table 1). Human and murine immunoglobulins were monoclonal proteins isolated from myeloma serum samples. The other preparations were polyclonal immunoglobulins purified from serum collected from non-immunized animals. A total of 19 human myeloma proteins belonging to the subclasses IgG1, IgG2, IgG3 and IgG4 were studied. These monoclonal components differed in electrophoretic mobility, type of light chain and type of protein A reactivity (precipitation/inhibition). Allotypes G1m(1), G1m(3), G2m(23), G3m(21) and G3m(5) were represented.

Type I receptors (staphylococcal protein A) were characterized by a restricted specificity interacting with human IgG1, IgG2 and IgG4, murine IgG2a, IgG2b and IgG3, bovine IgG2, ovine IgG2 and caprine IgG2 (Table 4). Murine IgG1, caprine IgG1, and equine IgG(ab) and IgG(c) demonstrated a weak protein A reactivity. Human IgG3, bovine IgG1, and ovine IgG1 were completely non-reactive.

Type II receptors (group A streptococci) interacted with all four human IgG subclasses and with polyclonal rabbit IgG. Murine, bovine, ovine and caprine IgG subclass preparations did not bind to type II receptors (Table 4). Equine IgG(ab) and IgG(c) were completely non-reactive. A weak reactivity was noted with equine IgG(T).

Type III receptors carried by Streptococcus equisimilis, Streptococcus dysgalactiae strains and human group G streptococci were characterized by a broad reactivity with positive binding of all human, bovine, ovine, caprine and equine IgG subclasses as well as three of four murine IgG subclasses (Table 4). Various bacterial species differed, however, in their capacity to bind murine IgG1. Small but definite amounts of murine IgG1 were bound to four out of 15 human group G streptococci. In contrast, no appreciable binding was noted to Streptococcus equisimilis nor to Streptococcus dysgalactiae strains. Low binding

Table 4. Specificities of defined types of immunoglobulin receptors for mammalian IgG subclasses.

Immunoglobulin	Type of immunoglobulin G receptor				
	I	II	III	IV	V
Human IgG1	+++	+++	+++	+	+++
Human IgG2	+++	+++	+++	- *	+++
Human IgG3	-	+++	+++	-	-
Human IgG4	+++	+++	+++	+	+++
Murine IgG1	+	-	+ **	NT	NT
Murine IgG2a	+++	-	+++	NT	NT
Murine IgG2b	+++	-	+++	NT	NT
Murine IgG3	+++	-	+++	NT	NT
Bovine IgG1	-	-	+++	-	-
Bovine IgG2	+++	-	+++	-	+
Ovine IgG1	-	-	+++	-	+
Ovine IgG2	+++	-	+++	-	+
Caprine IgG1	+	-	+++	-	+
Caprine IgG2	+++	-	+++	-	+
Equine IgG(ab)	+	-	+++	-	+
Equine IgG(c)	+	-	+++	+	+
Equine IgG(T)	-	(+)	(+)	(+)	(+)

Positive signs + and +++ denote low and high binding levels respectively.

(+) Weak, atypical reactivity

NT Not tested

* One of four myeloma proteins showed reactivity

** Four of 15 strains of human group G streptococci were reactive

levels were noted with equine IgG(T). Uptake of equine IgG(ab) and IgG(c) but not IgG(T) could be inhibited with polyclonal human IgG (Fig 1 in paper VI). The IgG(T) binding is therefore different from the regular Fc mediated immunoglobulin binding.

Type IV receptors (β -hemolytic bovine group G streptococci) showed a restricted specificity interacting mainly with human IgG1 and IgG4. A low reactivity was noted with equine IgG(T). All other mammalian IgG subclasses were non-reactive.

Type V receptors (Streptococcus zooepidemicus) demonstrated a protein A-like reactivity with positive binding of human IgG1, IgG2 and IgG4 but not of IgG3. However, type V receptors differed markedly from protein A in terms of affinity for other mammalian subclasses (Table 3 in paper IV).

The binding pattern of bacterial immunoglobulin G receptor types I-V are summarized in table 4.

E. Subclass related differences in receptor affinity

(Paper V and VI).

IgG subclasses are defined by small structural differences in the constant portion of the gamma chain. Structural alteration at or near the site on the immunoglobulin molecule which is involved in the interaction with bacteria could affect the binding property. The question of subclass related differences in receptor affinity was studied with isolated IgG subclass preparations made monomeric by column chromatography on Sephadex G-200. Purified IgG preparations were tested for their capacity to compete with isotope labelled IgG in a standardized assay.

Four human myeloma proteins representing the four human IgG subclasses were tested for their capacity to interact with streptococcal IgG receptors type II (group A streptococci, strain A-1) and type III (group G streptococci, strain G-148) (Fig 5 in paper V). Both receptor types interact with all four

human IgG subclasses. In the A-1 test system the IgG1 myeloma protein was more competitive than the three other myeloma proteins representing IgG2, IgG3 and IgG4. In the G-148 system the IgG1 and IgG2 preparations showed a higher degree of competition than the IgG3 and IgG4 myeloma proteins. These differences suggest that the receptors carried by the two test strains differ in their affinity for human IgG subclasses. The experiments were, however, limited to only one myeloma protein of each subclass. A larger collection of myeloma proteins has to be studied before the question of subclass-related differences in receptor-affinity can be solved.

Bovine, ovine and caprine IgG subclasses interact with receptor types I and III but not with type II receptors. The affinity of non-human IgG subclasses for type I and type III receptors was explored as described for human IgG subclasses (Fig 2 in paper VI). The IgG subclass preparations tested differed markedly in their capacity to compete with labelled human IgG1 for binding to two selected strains carrying type I and type III receptors. The competitive design of the test system made it possible to compare various immunoglobulin preparations with respects to receptor affinity. The absolute values of the affinity could not be obtained with this method but the relative affinity could be determined with polyclonal human IgG as a reference immunoglobulin.

The ruminant IgG subclass preparations differed in their capacity to compete with the labelled ligand for binding to the Staphylococcus aureus strain Cowan I (type I receptors). The reference immunoglobulin human IgG was the most potent competitor followed by ovine IgG2, caprine IgG2, bovine IgG2 and caprine IgG1. These data indicate that mammalian IgG subclasses may differ substantially in the degree of protein A reactivity.

The differences observed with the human group G strain G-148 (type III receptors) were less marked. Human IgG was a more potent inhibitor than non-human immunoglobulins. Ovine IgG1, bovine IgG1 and caprine IgG1 immunoglobulins were more inhibitory than the IgG2 subclasses in these three species. Thus the IgG1 subclass in ruminant species has a higher affinity than IgG2 immunoglobulins for type III receptors.

Allotypes are genetically determined antigens confined to the constant portion of the immunoglobulin molecule. A number of allotypes, referred to as Gm types, have been defined in humans (44, 115). They reflect minute structural variations often limited to a few amino acids and usually restricted to a single subclass. Human myeloma proteins of defined allotypes were purified, radio-labelled and the degree of binding to bacteria determined (Table 2 in paper V). No definite relationship between allotype and degree of immunoglobulin reactivity could be established.

F. Binding of isolated IgG fragments to bacteria

(Paper V).

Human polyclonal IgG and three IgG myeloma proteins were digested with papain. The proteolytic fragments were separated from undigested IgG by gel filtration on Sephadex G-100. Fab and Fc fragments were isolated by affinity chromatography performed with anti-Fab and anti-Fc specific immunosorbent columns. Purified fragments were radiolabelled and tested for binding to selected bacterial strains carrying defined receptor types (Table 3 in paper V). Fc fragments showed a high degree of reactivity equivalent to the uptake levels noted with intact IgG molecules. Fab fragments from two myeloma proteins did also demonstrate reactivity. Unlike the well established Fc-mediated binding this new reactivity was found to be restricted to human group G streptococci. The observed binding levels indicated that the Fab reactivity was of lower affinity than the Fc reactivity. Non-immune binding of intact IgG molecules seems therefore to be mediated by the dominating Fc reactivity of the immunoglobulin molecule.

G. Determination of the IgG binding capacity

(Paper V).

The maximal binding capacity of selected strains representing Staphylococcus aureus, group A and G streptococci was determined by allowing increasing amounts of human IgG to bind to a standardized bacterial suspension containing 5×10^8 bacteria (Fig 3 in paper V). The Staphylococcus aureus strain Cowan I was the most potent binder with a binding capacity of nearly 20 μg IgG when tested at an IgG concentration of 50 $\mu\text{g}/500 \mu\text{l}$. Group A (strain A-1) and group G (strain G-148) streptococci were capable of binding 5 and 10 μg IgG, respectively. Studies of several other strains confirmed that β -hemolytic human group C and G streptococci were capable of binding twice as much IgG as group A strains. The binding capacity of all streptococcal isolates studied was lower than the capacity of Staphylococcus aureus strains.

H. Kinetic aspects of the immunoglobulin binding

(Paper V).

Kinetic studies of the immunoglobulin binding were carried out with human IgG and selected bacterial strains carrying the major types of IgG receptors. Purified myeloma proteins made monomeric by gel filtration on Sephadex G-200 were used as ligands. The association event was explored in a modified binding assay using defined amounts of ligand and bacteria. The binding step was terminated after defined incubation times and the bound fraction determined. A time-dependent process with rapid association of the components was noted (Fig 4A in paper V). Staphylococcus aureus demonstrated the fastest uptake with an almost maximal binding within one minute. The binding to streptococcal receptors type II and III was somewhat slower with nearly complete binding within 2-3 minutes.

The IgG uptake to the bacterial surface is reversible. The dissociation of bound immunoglobulin was studied by first reacting radiolabelled IgG with bacteria in a minimal volume and then diluting the mixture several times with buffer. Bound immunoglobulin will dissociate until a new equilibrium is reached. When bound immunoglobulin was plotted against time an initial phase of rapid dissociation and a second phase with a much slower dissociation was observed (Fig 4B in paper V). Analysis of these data indicated that the dissociation process follows first order kinetics.

I. Estimation of the equilibrium constant of the IgG-receptor interaction (Paper V).

The affinity of the streptococcal immunoglobulin receptors for IgG was estimated by testing variable amounts of a human IgG1 myeloma protein for binding to defined bacterial suspensions. Bound and free fractions were measured and the data plotted as described by Scatchard (Fig 6 in paper V) (118). Strains A-1 and G-148 representing type I and type III receptors, respectively, demonstrated non-linear plots. The equilibrium constants derived from the linear section of the plots were 9.4×10^7 liters per mole for A-1 and 7.8×10^7 liters per mole for G-148. The equilibrium constant measured with the staphylococcal strains Cowan I was 5.1×10^7 liters per mole.

J. Relationship to other streptococcal reactivities (Paper IV).

Streptococci can bind several serum proteins other than immunoglobulins. These interactions include binding of human serum albumin to group C and G streptococci (72, 100), fibrinogen to group A, C and G strains (71), and haptoglobin to isolates carrying the T-4 surface antigen (62, 113). Most strains used in the present investigation have been tested in previous studies for binding of these

serum proteins. These data were compared with the results from the binding experiments performed with human IgG. The immunoglobulin reactivity was demonstrated independently of the other described reactivities. However, several strains were capable of binding more than one protein. Such strains were selected for studies designed to explore the relationship between the various binding sites on the bacterial cell surface. Increasing amounts of human polyclonal IgG, human serum albumin and human fibrinogen were mixed with radio-labelled human IgG and the binding of iodinated immunoglobulin to bacteria determined. Human IgG showed a dose-dependent inhibition. Human serum albumin and fibrinogen had no effect of the IgG uptake even at concentration high enough to completely saturate the binding sites for these proteins. The immunoglobulin molecule seems therefore to bind to a structure which is spatially separated from the components involved in the interaction with albumin and fibrinogen.

DISCUSSION

A. Definition of different IgG receptor types

Non-immune immunoglobulin reactivity is defined as an interaction involving immunoglobulin structures other than the antibody combining site. The present systematic investigations of many species of Gram-positive cocci confirm earlier observations that non-immune reactivity is a feature of Staphylococcus aureus and of group A, C and G streptococci (Table 2) (9, 33, 64). The reactivity previously reported in group B and D streptococci (8) and in Diplococcus pneumoniae strains (133) could not be substantiated by direct binding assays. The earlier reports were based on data obtained with the less reliable hemagglutination technique.

Five different patterns of reactivities could be defined by differences in specificity for mammalian IgG subclasses (Table 4). These reactivities are mediated by different types of binding sites, conceptually referred to as IgG receptor types I-V (Table 3). Receptor type I represents protein A and is found only in Staphylococcus aureus strains. This reactivity is characterized by a

restricted specificity for human, murine, bovine and ovine IgG subclasses (37, 68, 74, 81). Receptor types II-V are found in streptococci. Type II, IV and V receptors are confined to group A streptococci, β -hemolytic bovine group G strains and Streptococcus zooepidemicus strains, respectively. Type II receptors interact with all four human IgG subclasses and with polyclonal rabbit IgG. Type II receptors have no affinity for ruminant IgG subclasses. Type IV and V receptors are also limited in their reactivity with IgG subclasses (Table 4). Type III receptors have a wide distribution. They can be demonstrated in two group C streptococcal species and in human group G streptococci. They exhibit a broad reactivity with binding of nearly all IgG subclass preparations tested. It is interesting to note that there is a complete correlation between receptor type and the two proposed types of species in β -hemolytic group G streptococci (Paper II). Human and bovine group G strains are morphologically identical and demonstrate only minor differences in sugar fermentation tests (Paper II).

Heat treatment of bacteria at 80°C for 5 minutes did not affect the reactivity (Paper V). Digestion of bacteria with pepsin at suboptimal pH and with trypsin at 7.5 resulted in a marked reduction in specific binding capacity (Paper IV). Thus the IgG receptors are heat stabile components which are either proteins themselves or connected to the bacterial surface by a protein structure. Human serum albumin and fibrinogen had no inhibitory effect on the uptake of radio-labelled IgG to streptococcal strains carrying binding sites for these three serum components (Paper IV). Furthermore, immunoglobulin reactivity can be detected in individual strains independently of the capacity to interact with albumin, fibrinogen, aggregated β_2 -microglobulin, and haptoglobin (65, 69, 100). The IgG receptors in streptococci must therefore have a surface localization which is spatially separated from the binding sites for other human proteins. There is no definite correlation between immunoglobulin reactivity and type of M protein and T antigen complex (65).

A central point is the relationship between the various IgG receptor types which are currently defined by their immunoglobulin specificity. From a statistical standpoint the occurrence of five different types of IgG receptors in closely related bacterial species is very unlikely. A more logical explanation is that they have a common evolutionary origin, i.e. that they have evolved

during the evolution of bacteria from an original immunoglobulin binding structure. The defined IgG receptor types may therefore be structurally more similar than indicated by the differences in binding properties. The theory of a common evolutionary origin is consistent with the fact that each receptor type can be demonstrated only in one or a few closely related bacterial species. There are differences in reactivity between strains carrying the same receptor type (Papers I, IV and V). Intra-species variation in immunoglobulin reactivity has also been reported by other investigators (5). These observations can be explained by minor structural variation within each IgG receptor type.

B. Specificity of defined immunoglobulin receptors

Binding experiments carried out with purified Fab and Fc fragments derived from human monoclonal IgG myeloma proteins confirmed earlier reports that the streptococcal IgG reactivity resides in the Fc portion of the immunoglobulin molecule (Paper V) (6, 64). The fact that the Fc fragments were as effective inhibitors as intact IgG molecules indicates that the Fab portion is not involved in the binding of IgG. Isolated Fab fragments from two IgG myeloma proteins tested in direct binding assays showed a definite binding to streptococci carrying type III receptors (Paper V). This reactivity represents a new type of non-immune interaction normally concealed by the dominating Fc reactivity.

The interaction between staphylococcal protein A and human IgG Fc fragments has recently been studied by high resolution crystallography (19). These studies showed that the Fc-reactive fragment B of protein A provides two areas of contact with the Fc portion of the IgG molecule, one extensive hydrophobic area and another smaller area involving a few polar residues. Histidine 435, an external residue seems to be essential for the protein A reactivity. In the protein A negative IgG3 subclass this residue is exchanged for arginine (94, 148). Streptococcal receptor types II and III bind all four human IgG subclasses (Papers IV and V). Type II and III reactivities must therefore have a molecular basis different from that described for protein A. The interaction with the different receptor types can be mediated by distinct sites, overlapping sites or by the same site on the immunoglobulin molecule.

Unlabelled monomeric myeloma proteins were tested for their capacity to compete with a radiolabelled IgG preparation for binding to IgG receptors type II and III (Paper V). Individual myeloma proteins representing all four human IgG subclasses differed in their inhibitory capacity. The homogeneous nature of the myeloma proteins implies that the heterogeneity reflects differences in affinity for the defined IgG receptor type. Isotypic or allotypic determinants can be responsible for this variability. Similar observations have been described by other investigators (5). Hemagglutination inhibition studies performed with IgG binding structures extracted from group A streptococci indicated that some of the extracts had affinity for iso-allotypic markers (5).

Immunoglobulin reactive bacteria can bind immunoglobulin classes other than IgG. Protein A containing staphylococci can bind human IgA (45, 107, 117), IgM (41, 45, 80) and IgE (51, 55). Group A streptococci bind human IgA1 and IgA2 but not IgM (10, 99, 119). Human group C and G streptococci do not bind IgA nor IgM (99). The uptake of IgA to group A streptococci did not correlate with the binding of IgG and these two immunoglobulin reactivities are mediated by separate structures on the bacterial cell surface (99, 121).

Recent studies have shown that protein A in addition to the classical Fc reactivity also exhibits an alternative Fab mediated reactivity which can be detected in the major immunoglobulin classes in man and in many mammalian species (23, 24, 51, 97, 149, 150). This newly described Fab reactivity explains the low degree of protein A reactivity found when bovine IgG1 and ovine IgG1 were tested for binding to protein A-Sepharose. These two subclasses were previously considered protein A negative (37, 81).

C. Immunochemical aspects of the IgG-receptor interaction

Kinetic studies of the immunoglobulin reactivity demonstrated that immunoglobulins interacted in a well defined way with the specific binding sites on the bacterial cell surface. The immunoglobulin binding is characterized by an extremely rapid association of the components and a much slower dissociation event. The binding is reversible. The equilibrium constants of the IgG-receptor interaction derived from Scatchard plots were $5-9 \times 10^7$ liters per mole. The equilibrium constants for several antigen-antibody reactions have been determined and range from 1.0×10^4 to 6.5×10^{10} liters per mole (134). The interaction between IgG and IgG receptors present on mammalian cells have also been defined in terms of binding parameters. The complement component C1q binds to IgG with an equilibrium constant of approximately 10^8 liters per mole (79). The interactions of immunoglobulins with IgG receptors present on mammalian cells have also been characterized in terms of binding parameters. Binding constants for the interaction between IgE and Fc receptors of mast cells and basophil granulocytes range from 10^8 to 10^{10} liters per mole (14, 52). Murine IgG2a binds to murine macrophages with an affinity of about 2×10^7 liters per mole (142). Thus the affinity of human IgG for bacterial IgG(Fc) receptors type I and III is of the same order as that of low affinity antibodies and it is lower than the affinity for Fc receptors found on mammalian cells.

D. Use of immunoglobulin binding bacteria in immunological work

Fc-reactive bacteria bind immunoglobulins irrespective of their antigen specificity. The interaction is characterized by rapid binding and high affinity (Paper V). These properties are the basis for the use of such bacteria in immunological work.

Stabilized staphylococci have in combination with specific antisera been used for demonstration and identification of mammalian membrane antigens (15, 58, 59). Intact staphylococci have also been used for IgG absorption of patient

sera (1, 36, 57, 85, 127) and for detection of immune complexes (90, 141). There are, however, certain limitations to the use of Staphylococcus aureus organisms for absorption of human sera. Protein A combines with IgG1, IgG2 and IgG4 but leaves specific IgG3 antibodies present in the absorbed sample (72, 74). Furthermore protein A binds a minor fraction of both IgA and IgM (45, 143). Staphylococci are therefore not ideal particles for IgG versus IgM discrimination in serological work. Streptococci do not have these drawbacks and may be a valuable alternative to staphylococci. Group A streptococci, for example, bind all four human IgG subclasses, but not IgM (99). Some group A strains also bind IgA1 and IgA2 (99). Group C and G streptococci bind only the four IgG subclasses (99). By selection of appropriate bacterial strains differentiated absorption of serum samples can be performed (72, 82).

Protein A containing staphylococci have been used as "second antibody" in radioimmunoassays (28, 36, 56). One of the drawbacks of staphylococci in identification procedures using specific antibodies is the simultaneous binding of immunoglobulins present in the test sample. In combination with specific rabbit antibodies with Fc-reactivity for group A streptococci, such bacteria can be used in mouse models without interference of non-immune mouse immunoglobulin binding. Another obvious limitation in the use of staphylococci is the poor reactivity for ruminant immunoglobulins, antibodies often used in radioimmunoassays. The protein A negative IgG1 subclass in cattle constitutes approximately 45 per cent of the total IgG (3, 145). Human group C and G streptococci with their capacity to bind both IgG1 and IgG2 in ruminant species do not have this drawback. Thus selected streptococcal strains with defined immunoglobulin binding properties appear to offer substantial advantages compared with the currently used staphylococcal organisms.

Purified IgG receptors have many applications in immunology. Staphylococcal protein A linked to a Sepharose matrix has been used to isolate immunoglobulin classes and subclasses from serum samples (4, 25, 36, 48, 106). Iodinated protein A has been employed in immunoassays as a general immunoglobulin reagent (77, 78). Ferritin-conjugated protein A has served as an immunocytochemical reagent for electronmicroscopy (137). Enzyme labelled protein A was found to be a valuable tool for histochemical localization of antigens (20). Streptococcal IgG receptor type III with its broad reactivity with mammalian IgG present a valuable alternative to the currently used protein A.

E. Effects on the host-parasite balance

Several mammalian proteins are now known to interact specifically with bacterial surface structures. Immunoglobulin G (Papers I-IV, 31, 33, 64), IgA (10, 45), IgM (45), IgD (30), IgE (55), serum albumin (100), haptoglobulin (62, 113), fibrinogen (71), fibronectin (75) and complement component C1q (84, 112) are all capable of direct binding to bacterial cells. Specific interactions have also been noted with aggregated β_2 -microglobulin (69) and with heavy chains of HLA antigens functionally aggregated on liposomes (60). Recent studies have shown that Semliki forest virus can bind to major histocompatibility antigens on the mammalian cell surface (47). The biological significance of all these interactions between host proteins and microorganisms has not been studied.

Schistosoma mansoni, a human parasite residing in the intestinal blood vessel system of the host, can bind specific host proteins such as blood group substances (11) and histocompatibility antigens to its surface (123). Receptors with specificity for IgG(Fc) and β_2 -microglobulin have recently been identified on the tegumental surface of the parasite (136, 140). Acquisition of host-derived proteins seems to enable the parasite to evade the immune response of the host and in this way escape subsequent destruction (2). In analogy, uptake of mammalian proteins by pathogenic bacteria could provide a mechanism to resist normal disposal. Binding of host proteins could also affect the host-parasite balance in more direct ways. Recent studies have shown that uptake of IgG and serum albumin to bacteria results in altered surface properties (98). The microbial surface plays an important role in the interaction with phagocytic cells (128). Alteration of surface properties could therefore represent a mechanism to influence the host-parasite relationship. Furthermore, binding of immunoglobulins could initiate complex biological effects. Protein A, for example, can activate the complement system (67, 131). The multitude of host-bacteria interactions and their complexity must be fully recognized when experiments are designed to study the biological meaning and the survival value of such reactivities.

GENERAL SUMMARY

Staphylococcus aureus, and group A, C and G streptococci bind mammalian immunoglobulin G in a non-immune way. This reactivity is mediated by bacterial surface structures conceptually referred to as IgG(Fc) receptors. There are five different receptor types defined by their specificities for mammalian IgG subclasses. Each receptor type is confined to one or a few bacterial species.

Type I receptors, protein A, is found only in Staphylococcus aureus strains. Type I receptors interact with human IgG1, IgG2 and IgG4, murine IgG2a, IgG2b and IgG3, bovine IgG2, ovine IgG2 and caprine IgG2. Murine IgG1, caprine IgG1, and equine IgG(ab) and IgG(c) are weakly reactive. Type II receptors found in group A streptococci interact with all four human IgG subclasses but not with murine nor with ruminant IgG. Type III receptors are carried by Streptococcus equisimilis, Streptococcus dysgalactiae and human group G streptococci. Type III receptors are characterized by a broad reactivity with binding of all human, bovine, ovine, caprine and equine IgG subclasses as well as murine IgG2a, IgG2b and IgG3. Type IV receptors present on bovine group G streptococci bind small but significant amounts of human IgG1 and IgG4. Type V receptors confined to Streptococcus zooepidemicus strains are protein A-like. Type V receptors interact with human IgG1, IgG2 and IgG4 but differ from protein A in their affinity for bovine, ovine and caprine IgG subclasses.

Mammalian immunoglobulins interact with bacterial IgG(Fc) receptors in a well defined manner. The non-immune reactivity is mediated by structures found in the Fc portion of the immunoglobulin molecule. The interaction is characterized kinetically by a very rapid association phase and a slow dissociation phase. The equilibrium constants of the IgG receptor binding were 9.4×10^7 and 7.8×10^7 liters per mole for type II and type III receptors respectively.

The binding of immunoglobulins to suspended bacteria is completed after a few minutes of incubation. Suspensions of human group C and G streptococci containing 5×10^8 organisms are capable of binding approximately 10 μ g human IgG when tested at an IgG concentration of 50 μ g per 500 μ l. The binding capacity

of group A streptococci is on the average 5 μ g human IgG. In comparison the Staphylococcus aureus strains Cowan I is capable of binding nearly 20 μ g human IgG.

The IgG(Fc) receptors in streptococci are heat stable structures susceptible to digestion with proteolytic enzymes. The IgG receptors have a surface localization which is spatially separated from the binding sites for human serum albumin, fibrinogen and aggregated β_2 -microglobulin.

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Erling B. Myhre

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APPENDIX
Publications I-VI

I

Heterogeneity of Nonimmune Immunoglobulin Fc Reactivity Among Gram-Positive Cocci: Description of Three Major Types of Receptors for Human Immunoglobulin G

ERLING B. MYHRE AND GÖRAN KRONVALL*

The Department of Medical Microbiology, Sölvegatan 23, Lund, S-223 62 Sweden

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Two hundred and thirty strains of various gram-positive cocci were tested for quantitative, nonimmune binding of radiolabeled human polyclonal immunoglobulin G (IgG). The majority of coagulase-positive staphylococci and streptococci belonging to serogroups C and G showed a high uptake of IgG. The binding of immunoglobulin to group A streptococci was considerably less, with a number of strains completely negative. None of the pneumococcal or the group B or D streptococcal strains displayed any binding capacity. Heterogeneity of the IgG reactivity of various reactive strains was studied in an inhibition assay using 10 different animal serum pools. Three different inhibition patterns were seen, each of them revealing a striking degree of homogeneity within single bacterial species. *Staphylococcus aureus* and group A streptococci, respectively, constituted two homogeneous groups which differed markedly from each other and from C and G streptococci. No differences were observed between group C and G streptococci. Based on the profound differences between these homogeneous groups, three major types of Fc receptors could be defined. Type I and II Fc receptors were found on *S. aureus* and on group A streptococci, respectively. Fc receptor type III represented the immunoglobulin-binding structure of both group C and G streptococci.

Most strains of *Staphylococcus aureus* carry a surface component, protein A, with the capacity to combine with the Fc part of immunoglobulin molecules (12, 21, 24, 25). In humans, immunoglobulin G (IgG) subclasses 1, 2, and 4 are reactive (24). Strong evidence suggests that subclasses of human IgA and IgM are reactive as well (15). Furthermore, immunoglobulins from various mammalian species can also interact with protein A (23).

The immunoglobulin Fc-binding capacity of protein A has already been utilized in various fields of immunological technology. These methods include the coagglutination method for serological identification of microorganisms (4, 9, 19, 31), the use of stabilized staphylococci as a separation reagent in radioimmunoassays (10, 17) as antibody adsorbent (7), and labeled protein A as a marker for cell-bound immunoglobulin (1, 2, 7, 8, 13). The immunoglobulin reactivity of protein A might also play a role in host-parasite relationships (11).

Group A, C, and G streptococci carry an Fc-binding structure analogous to protein A (20). Sensitized sheep cell agglutinating properties have been reported to occur in group B and D streptococci (5) and in pneumococci (29). Ex-

vious studies have indicated differences in the immunoglobulin reactivity of staphylococci and group A, C, and G streptococci (20). The purpose of the present investigation was to study this suggested heterogeneity of Fc reactivities in a larger number of bacterial isolates. The possible existence of immunoglobulin-binding surface components with different specificities might expand the potentials of the use of such Fc reactivities in immunological techniques. Further clarification regarding immunoglobulin specificity and homogeneity within different bacterial species might also provide the basis for studies to determine the biological significance of nonimmune immunoglobulin reactivity.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. A total of 230 unselected strains were included in the present study: 30 strains of *S. aureus*, 40 strains of *Streptococcus pneumoniae*, and 30 group A, 40 group B, 30 group C, 30 group D, and 30 group G streptococci. All strains were isolated from routine specimens of human origin. Lancefield grouping of the streptococci was performed using the coagglutination method (4). A protein A-negative staphylococcal strain, Wood 46, served as a nonreactive control strain. Staphylococci were grown in tryptone broth,

and pneumococci and streptococci were cultured in Todd-Hewitt broth. After 16 h of incubation at 37°C, the bacteria were harvested and washed twice in phosphate-buffered saline, pH 7.2, containing 0.02% sodium azide. The optical density at 540 nm was measured, and the bacterial concentration was calculated from a standard curve and adjusted to 10^9 organisms per ml.

Radiolabeling of human IgG preparation. Freeze-dried, pooled human immunoglobulin was purchased from AB Kabi (batch no. 33574). Labeling with ^{125}I was performed using the chloramine-T method (27). Five milligrams of IgG (1 mg/ml in phosphate-buffered saline, pH 7.4) was mixed with 0.5 mCi of carrier-free ^{125}I in a beaker cooled in an ice bath. After addition of 50 μl of chloramine-T (5 mg/ml), the reaction was allowed to proceed for 8 min under stirring. The labeling process was stopped by addition of 100 μl of $\text{Na}_2\text{S}_2\text{O}_5$ (5 mg/ml). Free, unreacted isotope was removed by gel filtration on a Sephadex G-25 column. After determination of the protein concentration with a modified Folin method (26), human serum albumin (0.05% final concentration) was added to prevent nonspecific adherence of IgG to the test tube wall.

Radioimmunological assays. (i) Quantitation of IgG binding. All bacterial strains were tested for maximum uptake of radiolabeled IgG. These tests were carried out in plastic tubes (70 by 11 mm; A/S Nunc, Roskilde, Denmark) by adding 2×10^8 bacteria (200- μl suspension containing 10^9 test organisms per ml) to 0.5 μg of labeled IgG. After 1 h at room temperature, 2 ml of phosphate-buffered saline containing 0.02% sodium azide and 0.05% Tween 20 was added to each tube and the bacteria were spun down. The radioactivity in the pellet was measured in a Selectronic gamma spectrometer, and the uptake is expressed as percentage of total radioactivity added. An uptake of less than 10% was considered negative.

(ii) Inhibition assay. Highly reactive strains of staphylococci and streptococci belonging to serogroups A, C, and G (15 strains of each) were selected for use in an inhibition assay. In a pilot study, the inhibiting capacity of pooled normal human serum was determined. Increasing amounts of pooled normal human serum were mixed with 2×10^8 organisms, and 0.5 μg of labeled IgG was then added. In an extended study, the inhibiting capacity of pools of serum samples from the following species was determined: human, rabbit, rat, guinea pig, dog, cat, horse, pig, sheep, cow, and chicken. Based on the results from the pilot study, the streptococci and the staphylococci were then tested with 4- and 8- μl serum samples, respectively. These volumes represented the smallest amounts of human serum capable of giving complete inhibition in the assay. The test series using animal serum pools always included a determination of the maximum binding capacity of each strain. All test samples were processed as described for the uptake study. Nonspecific background adsorption was recorded in every experiment by testing the nonreactive strain Wood 46 simultaneously. Background counts were deducted from recorded uptake of IgG by the test strain. Inhibiting capacity of a serum sample was expressed as the difference between the maximum uptake and the uptake obtained in the inhibition assay.

To compare strains of different reactivity, it was necessary to express the degree of inhibition as percentage of maximum uptake of IgG. Less than 50% inhibition was considered negative.

RESULTS

Quantitative uptake of human IgG. Two hundred and thirty strains of gram-positive cocci of human origin were tested for binding of radiolabeled human IgG (Fig. 1). All 30 strains of *S. aureus* were highly reactive, with a capacity to bind 60 to 75% of the radioactivity in the IgG preparation. The 25 to 40% nonreactive radioactivity represents protein A-negative IgG-3 molecules, denatured IgG, and free iodine. There was rather little variation between different isolates, indicating a relatively homogeneous population with high affinity for IgG. None of the 40 pneumococcal isolates showed any reactivity. Additional tests on five pneumococcal strains using a larger excess of bacteria were also negative. Furthermore, 40 strains of group B streptococci and 30 strains of group D streptococci failed to demonstrate any binding of IgG. Among group A streptococci, 19 of 30 isolates were positive, with a varying degree of binding. The proportion of bound IgG did not exceed 40%. Twenty-five of 30 group C streptococci were capable of binding IgG. A high degree of reactivity was noted except for two strains. Group G streptococci showed a tendency toward a split population pattern. Twenty-five of 30 strains were strongly positive, with a binding capacity of 50 to 80% of added IgG, whereas the remaining five strains were negative.

Inhibition of IgG uptake by normal human serum. Human serum is capable of inhibiting the uptake of radiolabeled IgG to Fc-reactive strains. In a pilot study, the inhibiting capacity of pooled normal human serum was almost identical for strains of *S. aureus* and group A, C, and G streptococci (Fig. 2). Due to an apparent lower degree of reactivity, the group A curve was less steep. The uptake of IgG by all three groups of streptococci was reduced to one-half with serum samples of about 0.2 μl . Complete inhibition was obtained using 5 μl of serum. In contrast, staphylococcal strains required two to three times as much serum to achieve a comparable degree of inhibition. As the pilot study disclosed similarities between group C and G streptococci, three more strains of each of these groups were tested in a simplified inhibition assay using three serum samples. These inhibition curves were completely parallel.

Inhibition patterns using animal serum pools. To define possible differences in IgG-reactive structures, highly reactive strains were

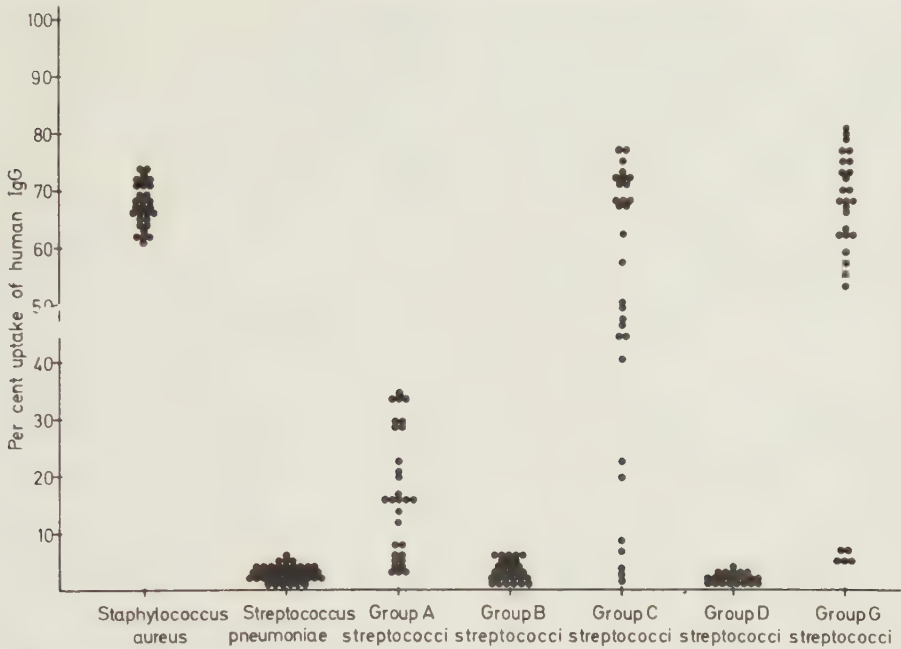


FIG. 1. Quantitative binding of human polyclonal IgG ($0.5 \mu\text{g}$) to strains (2×10^8 organisms) of *S. aureus*, *S. pneumoniae*, and streptococci belonging to serogroups A, B, C, D, and G. The binding capacity is expressed as percentage of added radiolabeled IgG.

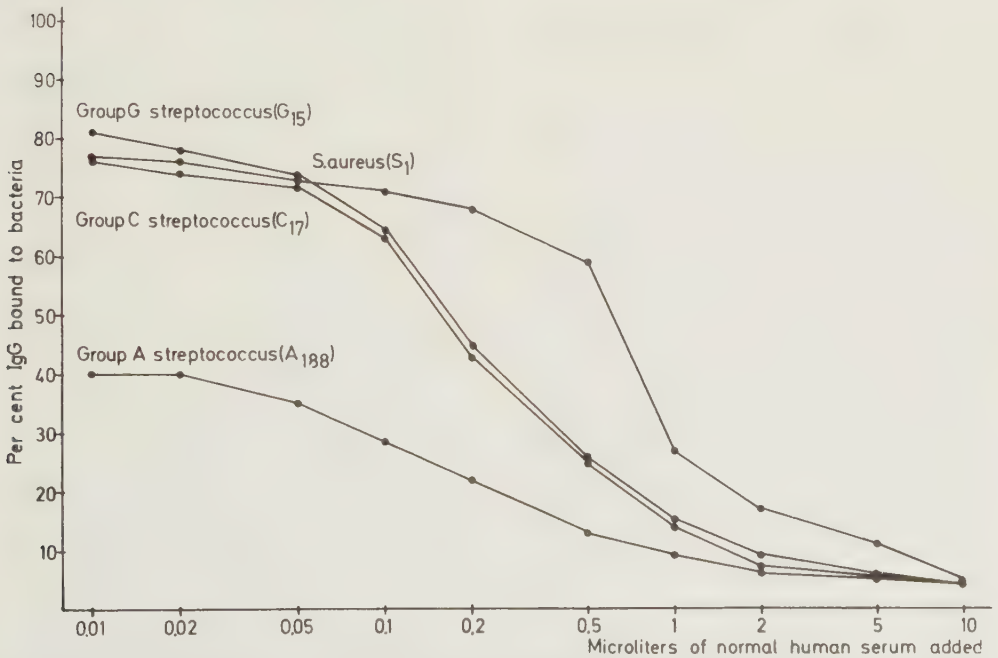


FIG. 2. Capacity of pooled human serum to inhibit the binding of radiolabeled IgG ($0.5 \mu\text{g}$) to representative strains (2×10^8 organisms) of *S. aureus* and of group A, C, and G streptococci. The uptake is expressed as percentage of radioactivity bound to the bacterial pellet.

tested in a special inhibition assay. In this assay, serum pools from 10 different animal species were allowed to compete with radiolabeled IgG for binding to the strains tested. Streptococcal and staphylococcal strains were analyzed using 4- and 8- μ l serum samples, respectively. To allow for comparisons of strains of varying degrees of IgG reactivity, the observed inhibition was expressed as percentage of maximum achievable inhibition. Fifteen strains of *S. aureus* were quite homogeneous, with a high degree of inhibition, when tested with human, rabbit, guinea pig, dog, cat, and pig sera (Fig. 3A). Rat, horse, sheep, cow, and chicken sera were negative.

Studies of 15 strains of group A streptococci revealed inhibition of IgG uptake by serum samples from humans, rabbits, and pigs. All other serum pools were negative in the inhibition assay (Fig. 3B). Group C and G streptococci (15 strains of each group) behaved as one uniform population regarding the inhibition patterns (Fig. 3C and D). Serum pools from humans, rabbits, guinea pigs, horses, pigs, sheep, and cows gave strong inhibition. Rat, dog, cat, and chicken serum pools were negative. The results of the inhibition studies using animal sera are summarized in Table 1.

DISCUSSION

The immunoglobulin specificity of protein A, the Fc-binding structure of the staphylococcal cell surface, is rather well characterized as far as the Cowan I strain (NCTC 8530) is concerned (12, 15, 22-24). Data on other strains of *S. aureus*, however, are not available, and relatively little is known concerning the streptococcal Fc receptor (3, 6, 20). The present study was designed to elucidate differences of Fc receptors of various bacterial species. Our approach involved the use of a battery of animal serum pools in a standardized inhibition assay. As immunoglobulins from related species differ slightly, they might be used to identify minor variations in the structures of immunoglobulin receptors. Highly reactive strains of *S. aureus* and of group A, C, and G streptococci were tested in this assay, and the inhibition profiles were plotted (Fig. 3A-D). These plots showed that the strains belonging to the same bacterial species displayed surprisingly little variation. Each bacterial species tested seemed to constitute a homogeneous group of organisms with identical types of Fc receptors. When the four different species were compared, distinct variations were observed. On the basis of these characteristic differences, three types of Fc receptors for human IgG were defined (Table 1). This

differentiation refers to the inhibition profiles obtained using serum pools from guinea pigs, dogs, cats, horses, sheep, and cows. Type I and II immunoglobulin Fc receptors are found on *S. aureus* and on group A streptococci, respectively. The type III Fc receptor is the immunoglobulin-binding structure of both group C and G streptococci. Further inhibition tests of six group C and G streptococci strongly supported the close similarity of the Fc-reactive structures among these strains. The present studies of IgG-binding structures of the bacterial cell surface in gram-positive cocci were performed using a well-established assay technique (6, 20, 22). The sensitivity of the test procedure depends on the ratio between quantity of IgG and the number of bacterial organisms (22). All tests were carried out with an excess of bacteria. Under these circumstances almost all of the reactive IgG molecules will bind to the bacterial surface when the binding affinity is high. The reactive fraction of the radiolabeled IgG preparation can thus be determined. A small quantity will be trapped nonspecifically in the bacterial pellet. This fraction can be estimated by testing a nonreactive strain (Wood 46). In our experiments, 5 to 8% of the labeled immunoglobulin preparation was bound nonspecifically. Based on this observation, strains were considered as reactive when they were able to bind more than 10% of added IgG.

In the present studies group B and D streptococci failed to reveal any IgG-binding properties. This is in contradiction to other studies (5). Nonimmune Fc reactivity has been reported to occur also in pneumococci (29). The report was based on studies performed with an agglutination procedure using sensitized sheep erythrocytes (32). In the present investigation we were unable to detect IgG-binding properties in any of 40 different isolates. As the isotope method used in our experiment is more reliable than the agglutination procedure, B and D streptococci as well as pneumococcal strains can be considered completely negative.

Several microbiological characteristics are common to group A, C, and G streptococci isolated from infections in humans. However, there are some factors indicating a position for group A strains separate from C and G streptococci. M-proteins are limited to A strains, with rare exceptions (14, 28). Group A streptococci are found only in humans, whereas group C and G strains are common causative agents in animal infections. Acute glomerulonephritis and rheumatic fever occur as postinfectious complications only after group A streptococcal infections (30). Our studies have added another feature

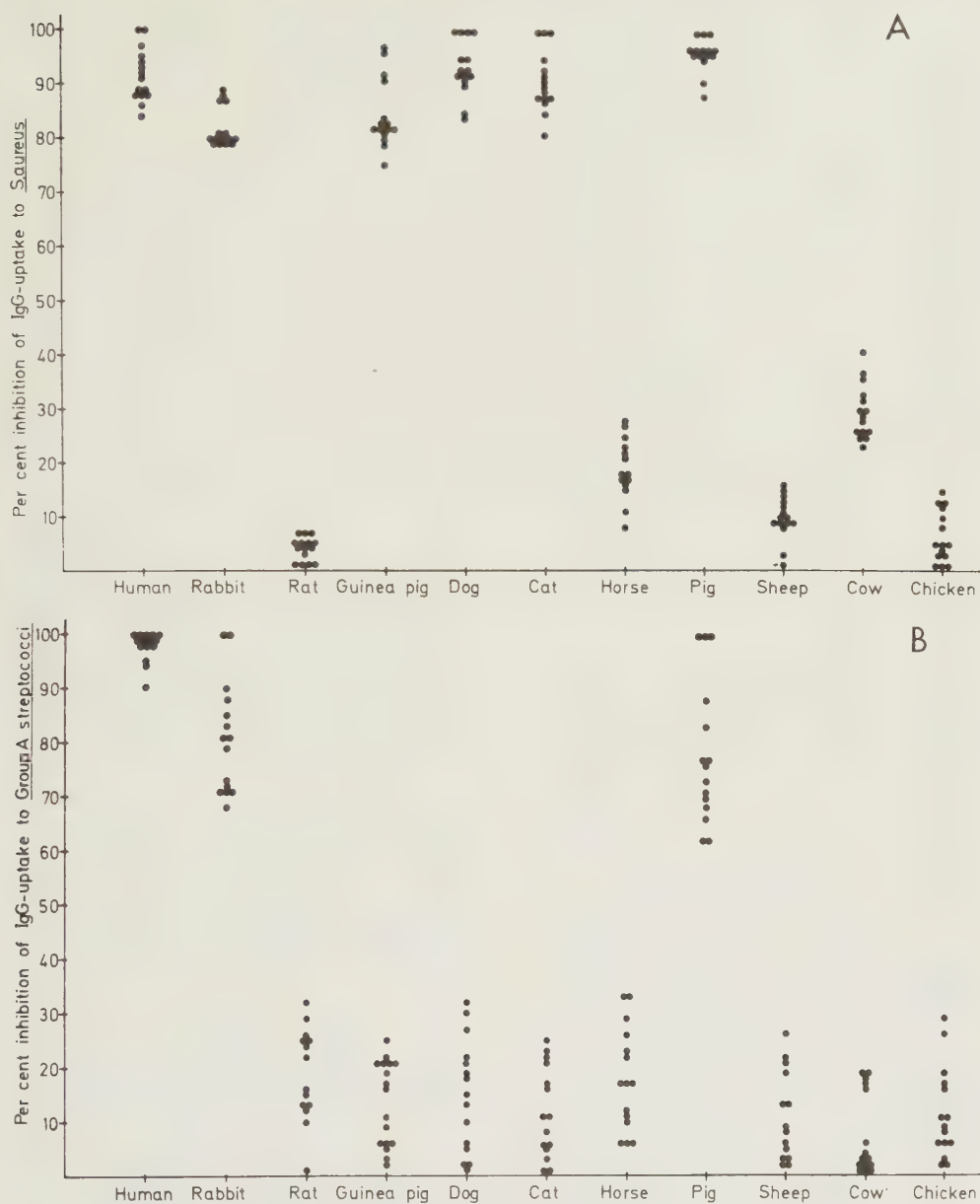


FIG. 3. Capacity of serum pools from various animal species to inhibit the uptake of radiolabeled IgG (0.5 μ g) to highly reactive strains (2×10^8 organisms). Inhibiting capacity is expressed as percentage of maximum obtainable inhibition. (A) Inhibition profile of 15 strains of *S. aureus* tested with 8- μ l serum samples. (B, C, and D) Inhibition profiles of 15 strains each of group A, C, and G streptococci tested with 4- μ l serum samples.

separating A strains from the other two groups. Both C and G streptococci carry a similar type of immunoglobulin Fc receptor, called type III, being markedly different from the type II Fc

receptor found on group A streptococci.

Protein A-carrying staphylococci are used for the separation of immunoglobulin-bound antigen from free antigen in radioimmunoassays

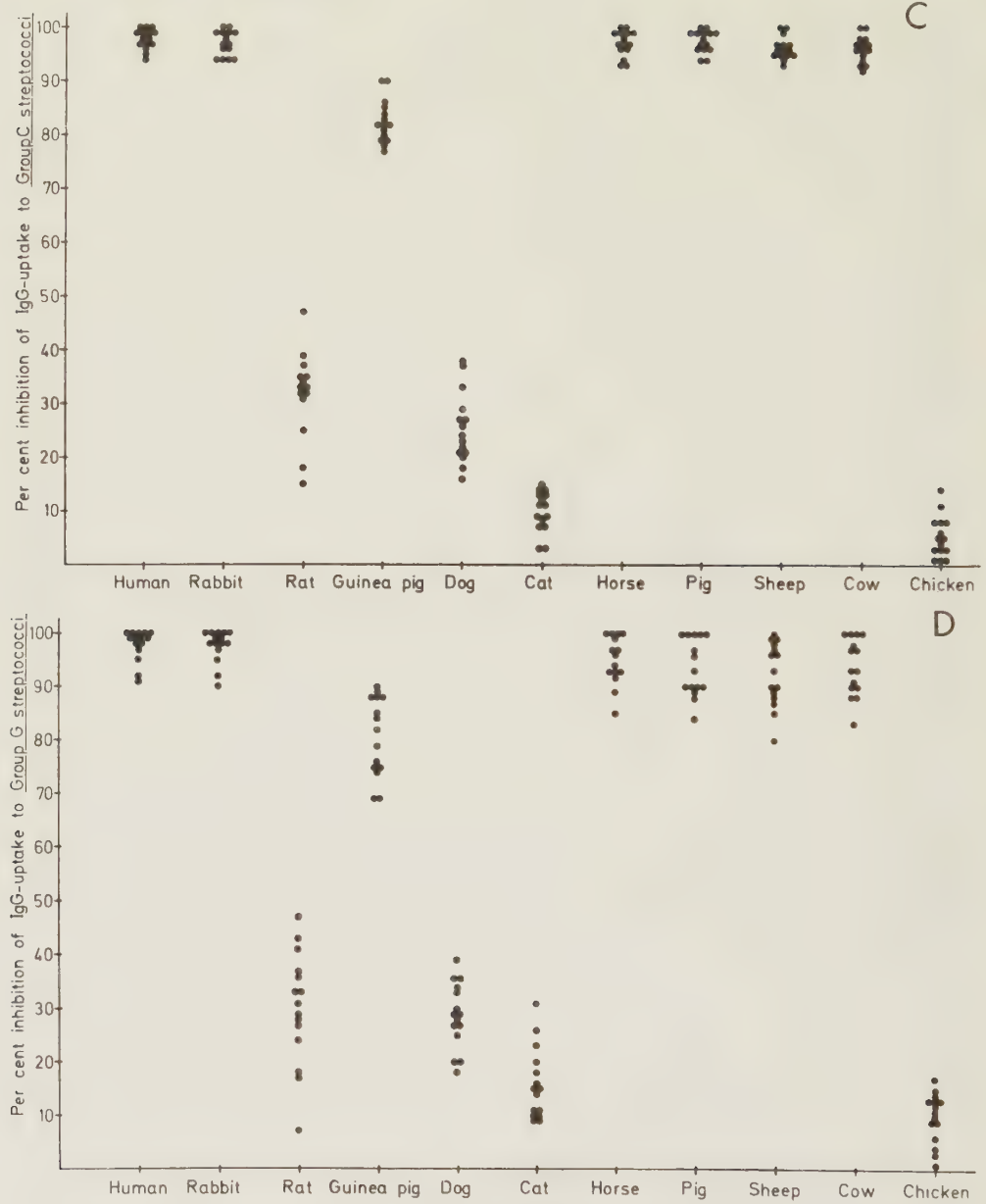


FIG. 3C and D.

(10, 17). Staphylococci coated with specific antibodies constitute coagglutination reagent for the identification of microorganisms and their soluble surface antigens (4, 9, 19, 31). Purified protein A can be labeled with fluorescein isothiocyanate, iodine, or ferritin and used for the detection of reactive immunoglobulin molecules

(1, 2, 7, 8, 13). Coupled to Sepharose 4B, protein A has been used for the isolation of IgG, particularly subclass IgG-3 (16). The use of protein A as exemplified is certainly far from fully exploited. The identification of similar but not identical Fc-binding structures could expand the present field of utilization. The demonstra-

TABLE 1. *Inhibiting capacity of various animal serum pools as studied in a standardized inhibition assay with 15 strains of each bacterial species^a*

Source of serum	<i>S. aureus</i>	Groups A streptococci	Group C streptococci	Group G streptococci
Humans	++	++	++	++
Rabbits	++	++	++	++
Rats	—	—	—	—
Guinea pigs ^b	++	—	++	++
Dogs ^b	++	—	—	—
Cats ^b	++	—	—	—
Horses ^b	—	—	++	++
Pigs	++	++	++	++
Sheep ^b	—	—	++	++
Cows ^b	—	—	++	++
Chickens	—	—	—	—
IgG Fc receptor type	I	II	III	III

^a ++, Inhibition exceeding 50%; —, inhibition less than 50%.

^b Differentiating serum sample.

tion of three different types of Fc receptors in the present study may therefore represent a significant step towards the definition of such similar Fc receptors. These receptors might, for instance, differ in their immunoglobulin class and subclass specificity, an aspect which would be of great interest to scientists concerned with the separation and characterization of immunoglobulins.

Attempts have been made to elucidate the biological significance of the IgG-binding property of protein A (11). Similar studies on Fc-reactive streptococci have not been reported. It is, however, quite clear that the Fc reactivity must be taken into account when aspects of acute streptococcal infections as well as the pathogenesis of postinfectious complications are studied.

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Immunoglobulin-Binding Structure on Bovine Group G Streptococci Different from Type III Fc Receptors on Human Group G Streptococci

ERLING B. MYHRE,^{1*} OLOF HOLMBERG,² AND GÖRAN KRONVALL¹

Department of Medical Microbiology, University of Lund, Lund, Sweden,¹ and National Veterinary Institute, Stockholm, Sweden²

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The immunoglobulin G (IgG)-binding capacity of 54 group G streptococci of human and bovine origin was investigated. Of 20 human strains, 17 carried a surface component which could combine with human IgG and bovine IgG₁ and IgG₂. Inhibition experiments with unlabeled human IgG and with a panel of animal sera revealed that the same surface component was involved in the binding of human as well as bovine immunoglobulins. Of 16 β -hemolytic bovine group G streptococci, 13 reacted with human IgG but not with bovine IgG₁ or IgG₂. This binding structure was different from the type III Fc reactivity found in human group G streptococci. All human strains, including the three IgG Fc-nonreactive strains, fermented trehalose, in contrast to all bovine β -hemolytic strains, which were negative. Immunoglobulin Fc reactivity is thus a feature not only of human strains but also of some bovine strains.

The streptococci comprise a large and biologically diverse group of microorganisms. The cell walls of hemolytic members contain serologically distinct carbohydrate antigens (17). These group-specific antigens separate strains into well-defined serological groups (17, 18). Group G streptococci may inhabit various parts of the human body and cause severe and even fatal infections (1, 5-7, 12, 28-30). Group G streptococci also colonize various domestic animals and may produce significant illness (2, 8, 10, 20, 23, 26). Serological studies of human as well as of animal group G streptococci have revealed antigenic heterogeneity of the bacterial cell surfaces and a number of serotypes in each group (21, 31).

Protein A-carrying staphylococci can react in a nonimmune way with the Fc portion of the antibody molecule (9, 14). This interaction has been extensively studied by using human immunoglobulin preparations (11, 16). Immunoglobulin G (IgG) from other mammalian species can also react with protein A (15, 22). Group A, C, and G streptococci possess an Fc-binding surface component analogous to protein A (13). This reactivity is mediated by two different types of reactive structures, one type carried by group A strains and the other found in both group C and G strains (27). Previous studies of human isolates have shown that most group G streptococci are reactive with a high IgG-binding capacity (27). Presence of this type of reactivity

among nonhuman isolates has not been investigated. Human and bovine group G streptococci form two groups of closely related organisms. The present studies were designed to compare these groups with respect to their IgG reactivity.

MATERIALS AND METHODS

Bacterial strains. A total of 54 group G streptococci were studied; 20 strains were isolated from clinical specimens of human origin, and 34 isolates were made from bovine milk samples. All human strains and 16 bovine isolates were β -hemolytic when grown on horse blood agar plates. The remaining 18 bovine strains were α -hemolytic. Lancefield grouping was performed by using the co-agglutination method (4).

Biochemical tests. Production of acid from the following carbohydrates was tested: adonite, arabinose, dextrin, dulcitol, glucose, inositol, levulose, maltose, mannitol, mannose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose, and xylose. The streptococci were grown in Todd-Hewitt broth for 6 to 8 h, and 2 drops of culture suspension was then transferred to tubes containing 1% carbohydrate in phenol red broth base (Difco Laboratories, Detroit, Mich.). All tests were read after 1 and 2 days of incubation at 37°C.

All human strains were quite uniform in their biochemical reactions, with production of acid from dextrin, glucose, levulose, maltose, mannose, salicin, sucrose, and trehalose. They did not split adonite, arabinose, dulcitol, inositol, mannitol, raffinose, rhamnose, sorbitol, or xylose. No differences were observed between reactive and nonreactive human isolates. The bovine strains fell into two different groups with distinct biochemical characteristics. The group contain-

ing the β -hemolytic bovine strains differed from the human isolate only by their inability to attack trehalose. The α -hemolytic organisms fermented dextrin, glucose, levulose, maltose, mannitol, mannose, salicin, sorbit, sucrose, and trehalose. No acid was produced from adonite, arabinose, dulcitol, inositol, raffinose, rhamnose, and xylose.

Radio-labeling of immunoglobulins. Freeze-dried pooled human IgG (lot 33574; AB Kabi, Stockholm, Sweden) and purified bovine IgG₁ and IgG₂ (Miles Laboratories, Inc., Elkhart, Ind.) were labeled with ¹²⁵I (code IMS 30; Radiochemical Centre, Amersham, England) by using the electrolytic iodination method (11). Protein concentrations were measured spectrophotometrically by using an absorbance ($E_{1\text{cm}}^{1\%}$) value at 280 nm of 13.5. Specific activities of labeled preparations were approximately 1 μ Ci per μ g of protein. To prevent nonspecific adsorption of labeled IgG to the test tube wall, human serum albumin was added to a final concentration of 0.05%.

Radio-immunological assays. (i) Quantitative IgG binding. All bacterial strains were cultured, washed, and suspended as reported previously (27). The IgG binding assay was performed in disposable polystyrene tubes (70 by 11 mm; AS Nunc, Roskilde, Denmark) by adding 2×10^8 bacteria to 0.1 μ g of labeled IgG. After 1 h at room temperature 2 ml of phosphate-buffered saline (pH 7.2) containing 0.05% Tween 20 was added to each tube. The bacteria were deposited by centrifugation, and the radioactivity in the pellet was measured in a gamma counter (LKB Rack Gamma 1270; Biotec, Stockholm, Sweden).

The IgG uptake was expressed as the percentage of total radioactivity added. Statistical computations were performed with a Hewlett-Packard 9815 A desk computer.

(ii) Inhibition assay. Increasing amounts of unlabeled IgG were mixed with 0.1 μ g of labeled immunoglobulin. Bacteria (2×10^8) of a highly reactive group G streptococcus of human origin (strain G-22) were added to each tube. The samples were then processed as described above for the IgG binding study. Similar inhibition experiments were also performed by using a panel of normal animal serum pools and bovine G streptococci for determining Fc binding type (27). The serum pools selected differ markedly in their inhibiting capacity of the uptake of human IgG to the various types of Fc-binding structures. They can therefore be used as reagents to detect differences in such bacterial surface structures. Recent studies of bovine immunoglobulins have shown that this capacity is due to both subclass differences in reactivity and serum concentrations of reactive immunoglobulins (Myhre and Kronvall, unpublished data).

Absorption of serum. Pooled normal human and bovine serum samples (0.1 ml) were absorbed at room temperature for 1 h with freshly harvested group G streptococci (0.05 ml of pelleted cells) grown in Todd-Hewitt broth. Serum samples were absorbed with a human, type III, Fc-reactive strain (G-32) and with a bovine, low-reactive strain (DG-10). To ensure complete absorption, the procedure was repeated once.

Immuno-electrophoresis. Pooled normal human serum, normal bovine serum, and absorbed serum samples were analyzed by immuno-electrophoresis in

a 0.6% agarose gel in 0.0075 M Veronal buffer, pH 8.6. Rabbit antiserum against human IgG was purchased from DAKO, Copenhagen, Denmark, and rabbit antiserum against bovine IgG was purchased from Cappel Laboratories, Downingtown, Pa.

RESULTS

Quantitative IgG binding. A total of 54 group G streptococci of human and bovine origin were tested for binding of radiolabeled human IgG and bovine IgG₁ and IgG₂ (Fig. 1A and B). Of 20 human strains, 17 were reactive with human IgG, binding 65 to 85% of the added amount (Fig. 1A). These strains also bound bovine IgG preparations (Fig. 1A). There was a difference, however, between the reactivities of the two subclasses. Bovine IgG₂ showed an uptake higher than that of human IgG ($P < 0.001$), whereas the binding of bovine IgG₁ was lower, ranging from 40 to 72% ($P < 0.001$). Three human strains were completely negative with the bovine immunoglobulin preparations but had a low uptake (9 to 11%) of human IgG.

Among the bovine group G streptococci a different pattern was obtained (Fig. 1B). No uptake of bovine IgG₁ or IgG₂ was noted for any of the 34 strains tested. Only 13 strains showed reactivity toward human IgG; all 13 were β -hemolytic group G streptococci. This group, with binding values of about 20% human IgG, was distinctly different from another group containing strains reacting only with 5% of added IgG. The binding observed in the first group could be inhibited by polyclonal human IgG and therefore does represent a low but definitely true Fc binding. In the second group no inhibition was seen by normal IgG, and the figures represent only a nonspecific trapping of immunoglobulin. Thus, all 18 α -hemolytic and the 3 β -hemolytic strains were Fc negative.

Individual strains of human group G streptococci were analyzed graphically for correlations with binding of the three different immunoglobulin preparations (Fig. 2A and B). The uptake of human IgG and bovine IgG₂ showed a linear correlation, with a correlation coefficient of 0.95. The comparison between human IgG and bovine IgG₁ binding revealed a similar correlation for individual strains, but with higher correlation coefficient values for nonlinear equations (correlation coefficient, 0.98; $\ln y = \ln a + bx$). This might reflect differences in binding affinities of the two types of immunoglobulins for the bacterial structures.

Inhibition of immunoglobulin uptake by human IgG. A strongly Fc-reactive human group G streptococcus (strain G-32) was used in inhibition experiments. Radiolabeled human IgG and bovine IgG₁ and IgG₂ were mixed with

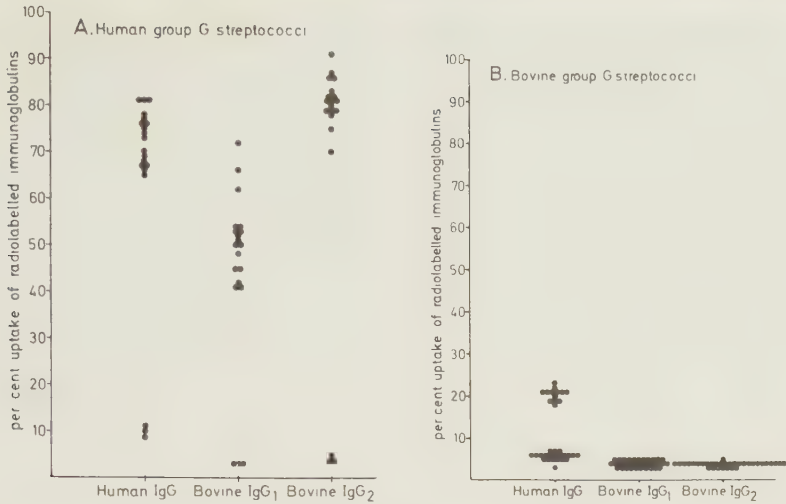


FIG. 1. Quantitative uptake of human IgG and bovine IgG₁ and IgG₂ by group G streptococci of human (A) and bovine (B) origin. The binding capacity is expressed as percent uptake of added radiolabeled IgG.

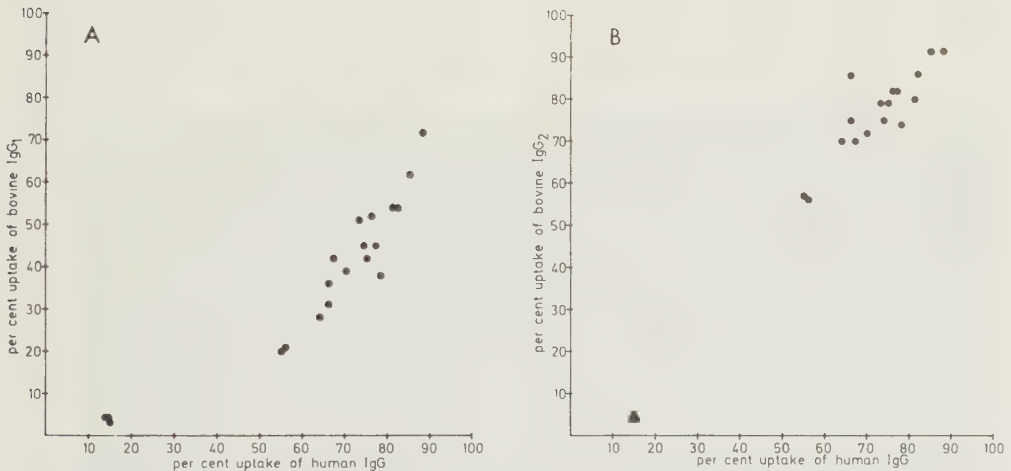


FIG. 2. Correlation between binding of human IgG and binding of bovine IgG₁ (A) and IgG₂ (B) to human group G streptococci.

varying amounts of normal human IgG (0.1 to 200 μ g) before the addition of 2×10^8 bacterial cells. The inhibition curves are shown in Fig. 3. Inhibition curves for uptake of human IgG and bovine IgG₂ were quite similar. Complete inhibition was seen after addition of about 200 μ g of human IgG. The lower affinity of bovine IgG₁ for Fc-reactive structures on human group G streptococci is reflected in the corresponding inhibition curve. Smaller amounts of unlabeled human IgG are required to inhibit the binding of bovine IgG₁.

Inhibition profile of bovine G streptococcus-human IgG interaction. Using a panel of animal sera at standard dilutions in inhibition assays, we have previously defined three types of Fc reactivities (27). Although the exact classes of inhibiting animal immunoglobulins are not yet defined, the serum pools have proved useful as probes to detect differences in the bacterial structures studied. Similar experiments were performed in the system described here, the bovine group G streptococcus-human IgG interaction. Human, rabbit, and guinea pig serum

pools were strongly inhibitory, whereas rat serum was negative. This conforms to type III Fc reactivity (27). However, serum pools from two Carnivores (dog and cat), one *Perrissodactylae* (horse), and three *Artiodactylae* (pig, sheep, and cow) differed in their inhibitory activity in the system as compared with the type III profile. Whereas cat serum was negative and horse serum was positive in type III inhibition, almost the opposite was found in the present system. Complete inhibition curves over a wide range of serum dilutions were therefore made with cat and horse serum (Fig. 4). Uptake of normal human IgG to the human group G streptococcus

strain G-44 was strongly inhibited by horse serum. Cat serum did not affect IgG binding. The uptake of IgG to the bovine group G streptococcus strain DG-10 was inhibited by both serum pools, but to a lesser extent by horse serum. Five more bovine G streptococci were tested for inhibition of IgG binding with 20- and 40- μ l samples of cat and horse serum. The inhibition by cat serum was in all cases stronger than the inhibition by horse serum. The experiments provide evidence for an IgG specificity of bovine group G streptococci different from the type III specificity of human group G streptococci.

Absorption of normal human and bovine

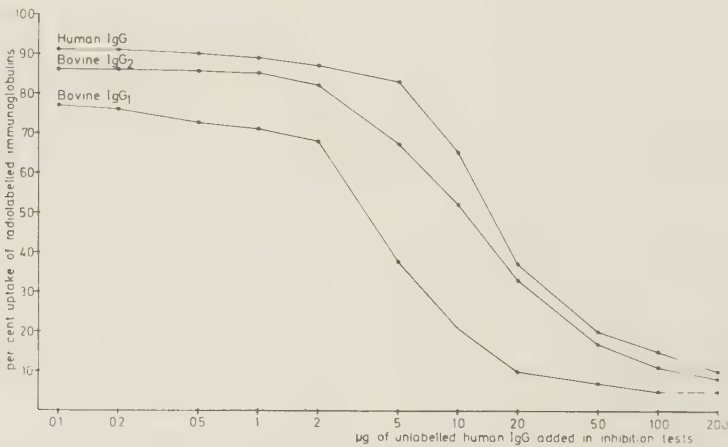


FIG. 3. Capacity of unlabeled normal human IgG to inhibit the binding of radiolabeled immunoglobulin preparations (human IgG and bovine IgG₁ and IgG₂) to a human group G streptococcus (strain G-22). The uptake is expressed as percent radioactivity bound to the bacterial pellet.

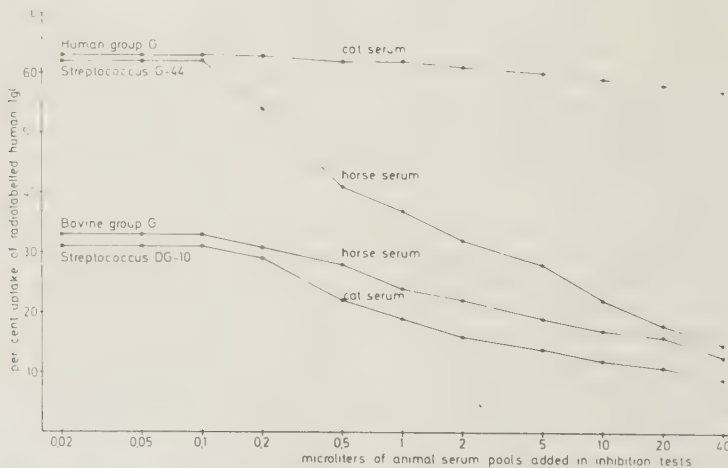


FIG. 4. Capacity of pooled normal horse and cat serum to inhibit the binding of radiolabeled human IgG to a human group G strain (G-44) and to a β -hemolytic bovine group G strain (DG-10).

serum samples. Further demonstration of the IgG-binding capacity of group G streptococci included absorption of normal human serum and bovine serum. One serum sample of each type was absorbed with an Fc-reactive human strain (G-32), and another was absorbed with a bovine strain showing a low but definite immunoglobulin binding (strain DG-10). Analysis of these samples by immunoelectrophoresis showed that the human strain removed all human as well as bovine IgG, whereas the bovine isolate gave only a slight reduction of human IgG (Fig. 5).

DISCUSSION

Group G streptococci represent closely related organisms causing infections both in human and animal hosts. The present study explored the IgG-binding capacities of three different series of group G streptococci: β -hemolytic human strains, β -hemolytic bovine strains, and α -hemolytic bovine strains. The latter strains are identical to the ones described by Fodstad (8). Quantitative binding assays demonstrated striking differences between strains of human and bovine origin (Fig. 1A and B).

Most human strains were capable of interacting with human IgG as well as with bovine immunoglobulin subclasses IgG₁ and IgG₂.

These two subclasses are antigenically distinct, although closely related. They are the most abundant immunoglobulins present in bovine serum (3). The reactivities of both bovine preparations confirm our previous observation that bovine serum inhibits the uptake of radiolabeled human IgG to group G streptococci (27). In contrast to human strains, β -hemolytic bovine group G streptococci bound only small amounts of human IgG and failed completely to interact with bovine IgG (Fig. 1B).

Several inhibition experiments were performed to detect differences in the immunoglobulin-binding surface structures. Panels of animal sera were used as previously described to obtain inhibition profiles for individual strains (27). Studies on human group G streptococci with labeled human IgG and bovine IgG₁ and IgG₂ revealed identical inhibition profiles characteristic of Fc reactivity type III. The β -hemolytic bovine strains, however, demonstrated a different inhibition profile, indicating a new type of binding structure restricted to this group of organisms (Fig. 4). This type of reactivity has a rather low binding potential for polyclonal human IgG. This may reflect a low binding affinity.

Inhibition experiments with a human group G strain showed that unlabeled human IgG could

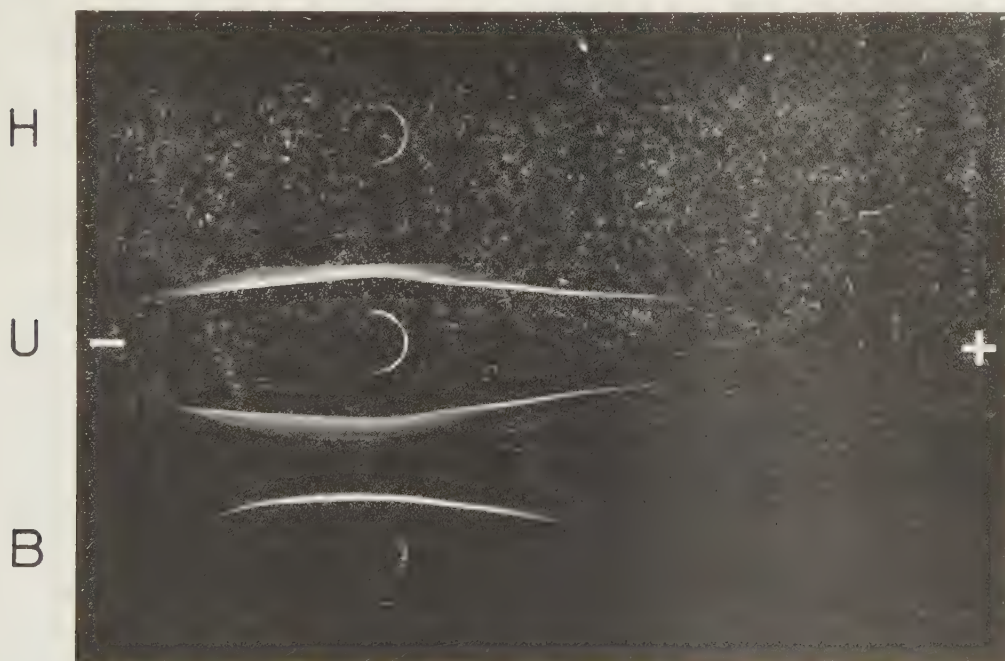


FIG. 5. Immunoelectrophoretic analysis of normal human serum after absorption with group G streptococci. H, absorption with an Fc-reactive human strain (G-32); B, absorption with a bovine low-reactive strain (DG-10); and U, unabsorbed serum as a control.

inhibit the binding of radiolabeled human and bovine IgG preparations (Fig. 3). Furthermore, the uptake by individual strains of human IgG correlated well with the binding of both bovine IgG subclasses (Fig. 2A and B). These two lines of evidence strongly suggest that the same surface component is involved in the binding of both human IgG and bovine IgG₁ and IgG₂.

Biochemical studies of the group G streptococci tested showed that the strains separated into groups with distinct fermentative properties. β -Hemolytic human and bovine strains which were morphologically indistinguishable differed in the utilization of trehalose. All human strains produced acid from this carbohydrate, whereas all bovine β -hemolytic strains were negative. This finding is in full agreement with earlier studies on both human (31) and bovine group G streptococci (10, 23). Trehalose fermentation as a distinguishing feature therefore seems to be well substantiated. The nonreactive human strains were biochemically identical to the Fc-reactive human strains, and these isolates are therefore definitely true human strains.

Reactive strains combine with the Fc portion of the antibody molecule (9). They can therefore bind immunoglobulin irrespective of their antigen specificity. Immunoelectrophoretic analysis of absorbed normal human serum samples showed that such strains are potent IgG-adsorbing agents (Fig. 5). Bovine immunoglobulins can also be effectively adsorbed out by reactive human group G streptococci. This supports our present finding that both bovine IgG₁ and IgG₂ subclasses bind to these strains. It is of interest that protein A can only absorb bovine IgG₂ (22).

The structure involved in the immunoglobulin reactivity of streptococci has not been identified. Two distinct types, however, have previously been described; one is restricted to group A strains, and the other is found in both group C and group G strains (27). This study adds a new type of Fc-binding structure present in β -hemolytic bovine group G strains.

The streptococcal surface is a complex structure carrying a number of antigens. Group A streptococci have well-defined protein components designated M, T, and R antigens (25). These antigens are not restricted to group A organisms but have also been reported in some group G strains of human origin (12, 19, 24). Nonhuman group G strains have distinct surface antigens which have been used for serotyping of bovine isolates (21). The nature of these antigens is to our knowledge uncertain, but it seems likely that they represent surface components similar to those carried by human strains. The present study, demonstrating immunoglobulin-binding structures not only in human strains but also in

bovine strains, suggests some similarity between these organisms.

ACKNOWLEDGMENTS

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Binding of Murine Myeloma Proteins of Different Ig Classes and Subclasses to Fc-reactive Surface Structures in Gram-positive Cocci

E. B. MYHRE & G. KRONVALL

Department of Medical Microbiology, University of Lund, Lund, Sweden

Myhre, E.B. & Kronvall, G. Binding of Murine Myeloma Proteins of Different Ig Classes and Subclasses to Fc-reactive Surface Structures in Gram-positive Cocci. *Scand. J. Immunol.* 11, 37–46, 1980.

Twelve different murine myeloma proteins were tested for binding to seventy Gram-positive strains belonging to group A, C and G streptococci and to *Staphylococcus aureus*. Group A streptococci, known to bind human IgG, were incapable of binding any of eight murine IgG immunoglobulins tested except for one strain that bound an IgG2b myeloma protein. In contrast, group C and G streptococci interacted with murine immunoglobulins of subclasses IgG2a, IgG2b and IgG3, and G strains also to a lesser extent with IgG1. Bovine and equine group C streptococci did not differ from human group C streptococci in their IgG reactivity. Staphylococcal strains showed a high reactivity with murine myeloma components of IgG subclasses 2a, 2b and 3 and a low but definite binding of an IgG1 myeloma protein. IgA myeloma protein S-122 interacted with nine of fifteen group A streptococci. This binding could not be inhibited by human IgG and the reactivity is thus different from Fc-mediated binding of immunoglobulins. One of three IgA myeloma proteins tested, TEPC 15, bound to staphylococci. The Fc specificity of this interaction was confirmed by chromatography on protein A-Sepharose and by inhibition studies using polyclonal human IgG. The protein A reactivity of this monoclonal protein was detected in IgA aggregates and absent in the monomeric form of IgA.

E. B. Myhre, Department of Clinical Microbiology, Sölvegatan 23, S-223 62 Lund, Sweden.

Protein A, a cell wall constituent of *Staphylococcus aureus*, interacts with the Fc portion of immunoglobulin molecules [7, 13]. Similar immunoglobulin-binding surface structures are found also on group A, C and G streptococci [12]. In previous studies we have described three major types of Fc receptors for human IgG [19, 20]. Type I and type II Fc receptors are found on *S. aureus* and on group A streptococci respectively. Fc receptors type III are carried by both human group C and group G streptococci. Protein A reactivity of murine immunoglobulins has been studied by others using a gel diffusion technique and affinity chromatography on protein A-Sepharose [14, 16, 17].

Protein-A-carrying staphylococci are used as solid-phase absorbents for isolation and identification of membrane antigens in combination with specific antisera [5, 10, 11], for detection of antigen-antibody complexes [2, 23], and as

a 'second antibody' in radioimmunoassays [1, 3, 9]. The potential use of other bacterial species possessing different types of immunoglobulin-binding structures prompted the present investigation. The study was designed to delineate the binding of murine immunoglobulin classes and subclasses to the major types of Fc-reactive surface structures previously defined in Gram-positive cocci. Group A streptococci were found to be incapable of Fc-mediated binding of mouse immunoglobulins. Such streptococci might therefore be used instead of protein A from *S. aureus* in studies in which mouse immunoglobulins might interfere with bacterial binding of antibodies from other species.

MATERIALS AND METHODS

Bacterial strains. Sixty human strains of *S. aureus*,

group A, group C (*Streptococcus equisimilis*) and group G streptococci, five equine group C (*Streptococcus zooepidemicus*) and five bovine group C (*Streptococcus dysgalactiae*) were tested for binding of murine myeloma proteins. The non-human isolates were all capable of binding human IgG. Five human group B streptococci served as Fc-non-reactive control strains. Lancefield grouping was performed with the coagglutination method [4]. Group C streptococci were characterized by morphology and by their fermentative action on sorbitol and trehalose [6].

Purified murine myeloma proteins. Myeloma proteins MOPC 21 (IgG1), UPC 10 (IgG2a), MOPC 195 (IgG2b), FLOPC 21 (IgG3), TEPC 15 (IgA), MOPC 315 (IgA) and MOPC 104E (IgM) were purchased from Litton Bionetics Inc. (Kensington, Md., USA). Myeloma proteins MOPC 213 (IgG2a), PC 5 (IgG2a), MOPC 141 (IgG2b), J 606 (IgG3) and S 122 (IgA) were obtained as previously described [14].

Radiolabelling of immunoglobulin preparations. Purified murine myeloma proteins were labelled with carrier-free ^{125}I (Code no. IMS 30, The Radiochemical Centre, Amersham, England). A modified chloramine-T method [18] was tried initially but was abandoned because labelled preparations showed low levels of binding to Fc-reactive test strains. Myeloma proteins labelled by mild electrolysis as described by Harboe & Fölling [8] demonstrated high binding capacities. This iodination method was therefore used throughout the studies. The specific activities of labelled preparations were approximately $1 \mu\text{Ci}/\mu\text{g}$ protein.

Radioimmunological assays. I. Quantitative binding assay. Bacterial strains were cultured, washed and suspended as previously reported [19]. Binding studies were performed as previously described with $0.2 \mu\text{g}$ labelled immunoglobulin protein and 2×10^8 bacterial organisms. Labelled preparations tested with Fc-negative control strains showed a non-specific background binding of 5–10% of the added radioactivity. Only PC 5, an IgG2a myeloma protein, was an exception, with a 20% background binding. The binding of labelled immunoglobulins to test tubes in the absence of bacteria was less than 1% of the added radioactivity.

Binding studies were also performed with a more sensitive test system in which the amount of radio-labelled myeloma protein was reduced to $0.04 \mu\text{g}$ and the number of bacteria increased to 2×10^9 .

II. Inhibition assay. Pooled normal human immunoglobulin (Lot no. 33574, AB Kabi, Stockholm, Sweden) was used for inhibition studies. The purity of the preparation was established by immunoelectrophoresis. A single precipitation arc was observed when tested at a concentration of 1 mg/ml against rabbit anti-human IgG and anti-human serum proteins obtained from Dakopatts (Copenhagen, Denmark). Inhibition experiments were performed by mixing $0.2 \mu\text{g}$ labelled myeloma protein with increasing amounts of human IgG. After addition of 2×10^8 bacterial cells, samples were processed as described for quantitative binding assays.

Dissociation studies. Radiolabelled myeloma proteins ($0.2 \mu\text{g}$) were allowed to combine with 10^9 bacteria of an *S. aureus* strain (S-1) and a group G streptococcus (G-148). The radioactivity of the bacterial pellets was determined, and the pellets were then suspended in 1.0 ml of KSCN solutions with molarities ranging

from 0.2 to 2.0. After 30 min at room temperature the bacteria were again deposited by centrifugation and the radioactivity of the second pellet measured. The amount of myeloma proteins still bound was expressed as percentage of radioactivity of the first pellet.

Affinity chromatography. Myeloma proteins MOPC 21 (IgG1), TEPC 15 (IgA) and MOPC 315 (IgA) were analysed for binding to protein A, covalently coupled to Sepharose CL-4B (Pharmacia Fine Chemicals). The binding capacity for human IgG was approximately 25 mg/ml of swollen gel. Samples containing between 12 and $21 \mu\text{g}$ of radiolabelled myeloma protein were applied to a 0.8×4.0 cm column and eluted at 4°C at a speed of 1 ml/h with PBS containing 0.02% sodium azide and 0.1% human serum albumin (HSA). After ten column volumes the absorbed fraction was eluted with 0.1 M glycine-HCl, pH 3.0, containing 0.1% HSA. The radioactivity of the collected 0.5 ml fractions was determined in a gamma counter.

Permeation chromatography. $100 \mu\text{g}$ radiolabelled myeloma protein TEPC 15 (IgA) was fractionated on a 1.6×70 cm column containing glyceryl-coated porous glass beads (G-CPG 350 Å, 220/400 mesh; Electro-nucleonics, Inc, Fairfield, N.J., USA). The column was eluted at a constant speed of 15 ml/h with PBS containing 0.02% sodium azide and 0.05% Tween 20. The radioactivity of 2.5 ml collected fractions was measured in a gamma counter.

RESULTS

Binding of murine myeloma proteins to bacteria

Twelve purified murine myeloma proteins labelled with ^{125}I were tested for quantitative binding to strains of *S. aureus* and group A, C and G streptococci. Several strains of each bacterial species were included because individual strains may differ in immunoglobulin binding capacity [19]. The specificity of the binding was tested in inhibition experiments in which increasing amounts of polyclonal human IgG was allowed to compete with radio-labelled myeloma proteins for Fc-binding bacterial structures. An uptake that could be inhibited by polyclonal human IgG was considered to represent an Fc-mediated binding.

IgG1. *Staphylococcus aureus* strains showed a low but definite binding of MOPC 21 ranging from 7% to 17% (Fig. 1A). In contrast, binding to group A and to group C streptococci did not exceed the level observed with Fc-negative control strains. Four out of fifteen group G streptococcal strains demonstrated reactivity with MOPC 21. The uptake of MOPC 21 to the staphylococcal strain S-1 and to the group G streptococcus G-41 could be completely

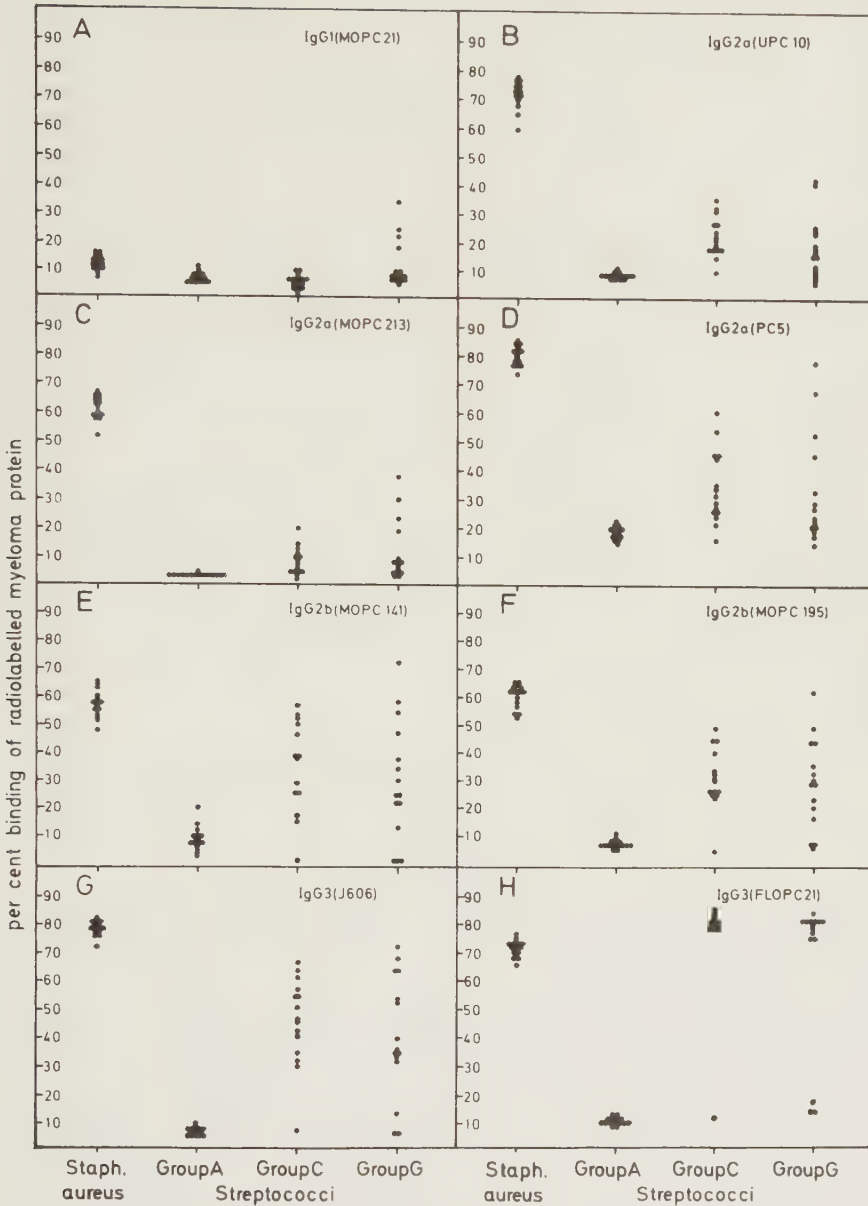


FIG. 1. Binding of purified IgG murine myeloma proteins to sixty human strains of *Staphylococcus aureus*, group A, C and G streptococci. Radiolabelled monoclonal components ($0.2 \mu\text{g}$) were allowed to bind to 2×10^8 bacteria. The binding capacity of individual strains is expressed as percentage of added activity.

inhibited with $10 \mu\text{g}$ polyclonal human IgG (Fig. 2A). The background binding to group A and group C streptococci was not affected by human IgG.

IgG2a. Three different myeloma proteins were studied: UPC 10, MOPC 213 and PC 5 (Fig. 1B, C and D). All three proteins showed a 55–85% binding to staphylococci. Group A

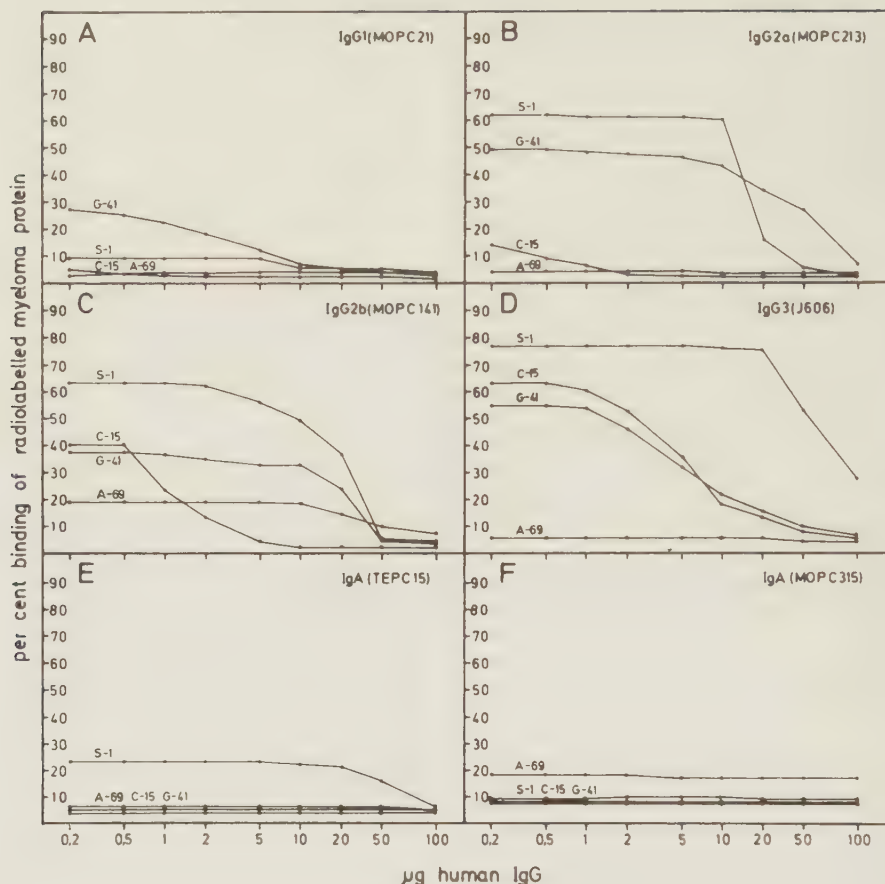


FIG. 2. Capacity of polyclonal human IgG to inhibit the binding of radiolabelled murine myeloma components to *Staphylococcus aureus* (S-1), group A (A-69), group C (C-15) and group G streptococci (G-41). Increasing amounts of human IgG were mixed with 0.2 µg murine myeloma protein and the binding to 2×10^8 bacteria determined.

streptococci bound 5–10% of UPC 10 and MOPC 213 and approximately 20% of PC 5. These levels were of the same magnitude as those recorded with non-reactive strains. Both group C and G streptococci presented a heterogeneous picture with positive binding levels ranging from 10% to 40% for UPC 10 and MOPC 213 and from 20% to 70% for PC 5. Inhibition experiments performed with MOPC 213 showed that the binding to the staphylococcal and to the group G strains was inhibited by 50–100 µg human IgG (Fig. 2B). The lower uptake by the group C strain was abolished by as little as 2 µg IgG. The group A strain included was non-reactive.

IgG2b. Staphylococci showed a 50–65%

binding of MOPC 141 and MOPC 195 (Fig. 1E and F). Group A streptococci bound approximately 10% of both preparations. A single group A strain, A-69, showed a 20% binding of MOPC 141 but was negative with MOPC 195. Fourteen group C and twelve group G strains were reactive, with uptake levels ranging from 15% to 70%. The remaining four isolates were negative. Complete inhibition of the binding of MOPC 141 to *S. aureus* and group G strains was seen with 50–100 µg human IgG (Fig. 2C). The binding of MOPC 141 to the reactive group A strain A-69 was also inhibited by these amounts of IgG. In contrast, 10 µg human IgG was sufficient to inhibit the binding to the group C strain.

IgG3. Preparations of J 606 and FLOPC 21 myeloma proteins demonstrated identical patterns of reactivity with positive binding of 70–80% to staphylococci and a background 10% to group A streptococci (Fig. 1G and H). The two myeloma proteins differed in their binding potentials towards group C and G strains. Fourteen group C and twelve group G strains showed an uptake of 80% of FLOPC 21. The same strains were also reactive with J 606 but with a spectrum of binding capacities ranging from 30% to 70%. Whereas 50–100 μ g IgG inhibited the binding of J 606 to the group C and G strains, the staphylococcal binding required higher amounts (Fig. 2D). The group A strain included was non-reactive according to the inhibition test.

IgA. Three myeloma proteins tested differed in their binding to both staphylococci and to group A streptococci (Fig. 3A, B and C). The staphylococci bound only 10% of MOPC 315 and S 122, in contrast to a 20% uptake observed with TEPC 15. Human IgG inhibited the binding of TEPC 15 to staphylococci (Fig. 2E).

Group A streptococci showed a higher uptake with MOPC 315 than with TEPC 15, but this binding could not be inhibited by human IgG (Fig. 2E, F). Nine group A streptococci bound 20–65% of S 122. This uptake could not be inhibited by human polyclonal IgG. None of the group C and G streptococci differed from the non-reactive control strains.

IgM. A single preparation tested, MOPC 104 E, did not combine with staphylococci nor with streptococci (Fig. 3D). A single group A streptococcus showed a 20% uptake, which, however, could not be inhibited by human IgG.

Ten equine (*Streptococcus zooepidemicus*) and bovine (*Streptococcus dysgalactiae*) group C streptococci were also studied. These strains did not differ from the human (*Streptococcus equisimilis*) group C streptococci in their Fc binding capacity. Thus reactivity was found with myeloma proteins of IgG subclasses 2a, 2b and 3. No binding of IgG1, IgA and IgM was observed.

Group G streptococci varied widely in their binding capacity. Plotting of the uptakes with

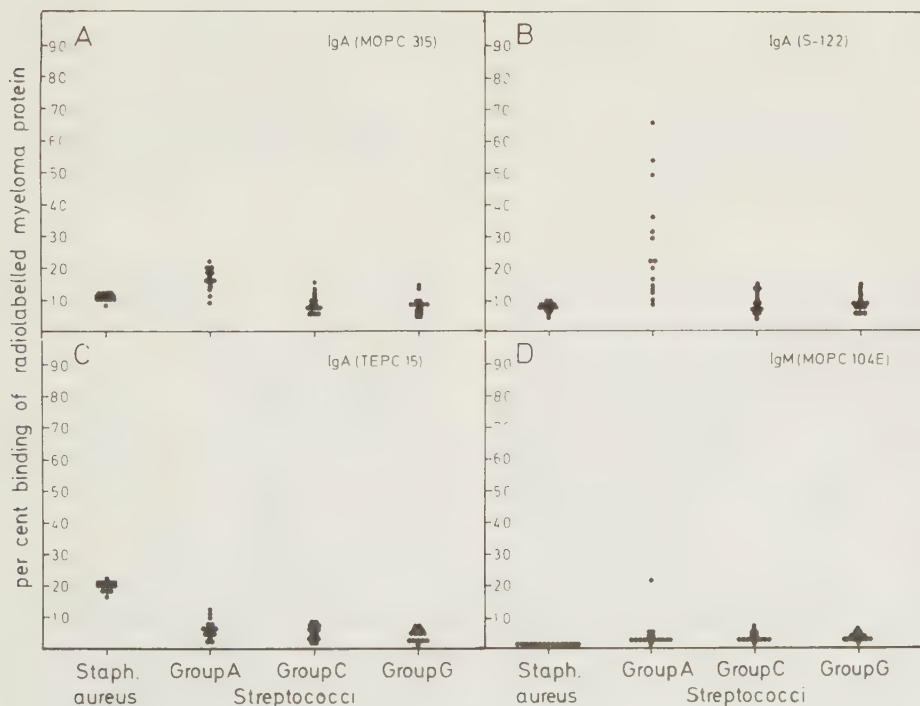


FIG. 3. Binding of purified murine myeloma proteins of IgA and IgM classes to sixty human strains of *Staphylococcus aureus* and group A, C and G streptococci.

different myeloma proteins revealed that the degree of reactivity was a constant property of the individual strain. Strains binding strongly one myeloma protein were also the most potent binders of other reactive preparations.

The low levels of binding observed with myeloma proteins MOPC 21 (IgG1) and TEPC 15 (IgA) might reflect a binding of low avidity. Binding studies were therefore performed with a more sensitive assay system using 2×10^9 bacteria. The uptake of MOPC 21 to an *S. aureus* strain (S-1) increased from 13% to 32% and that to a group G streptococcus (G-148) from 31% to 61%. TEPC 15 showed only a slight increase in binding to S-1 and no definite binding to G-148 even when these tests were performed with the higher numbers of bacteria.

Binding of murine myeloma proteins to protein A-Sepharose

In binding studies performed with whole

bacterial cells the myeloma proteins MOPC 21 (IgG1) and TEPC 15 (IgA) showed a low degree of binding to *S. aureus* strains. The specificity of these weak interactions was further investigated by affinity chromatography on protein A-Sepharose. MOPC 315, an IgA myeloma protein was included as a protein-A-negative preparation. In this test system both MOPC 21 and TEPC 15 demonstrated a high level of reactivity, in contrast to MOPC 315, which was completely non-reactive (Fig. 4). Analysis of collected fractions showed that 94% of IgG1 MOPC 21 and 72% of IgA TEPC 15 were eluted with 0.1 M glycine-HCl, pH 3.0 (Table I).

Elution of bacteria-bound myeloma proteins

Radiolabelled myeloma proteins bound to an *S. aureus* strain (S-1) and to a group G streptococcus (G-148) were dissociated using KSCN solutions of different molarities. MOPC 21 (IgG1) bound to S-1 and to G-148 was eluted

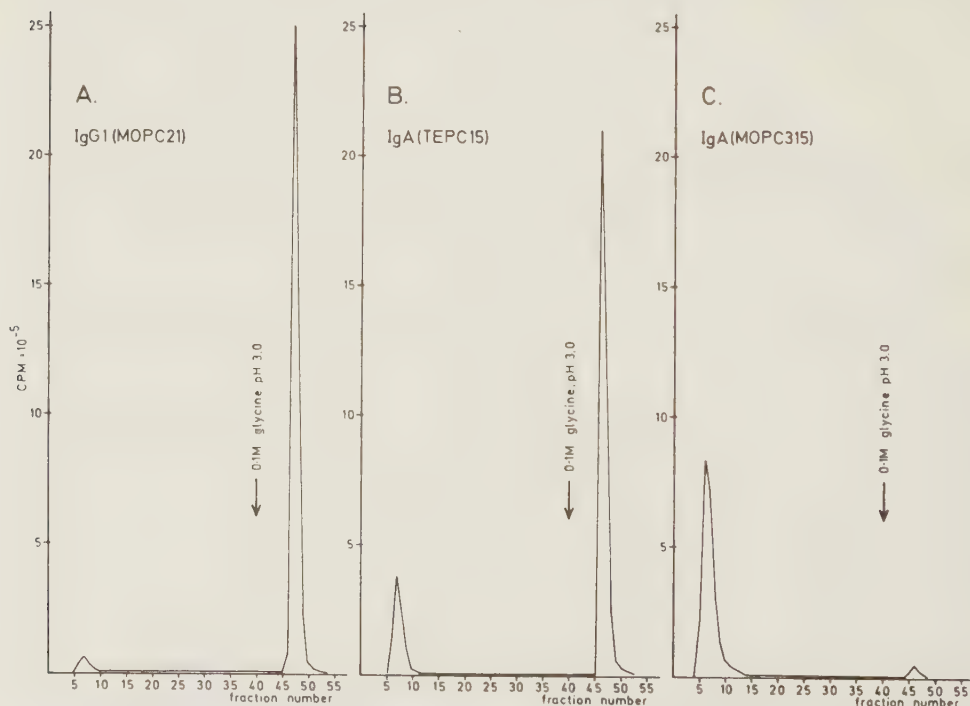


FIG. 4. Affinity chromatography of radiolabelled murine myeloma protein MOPC 21 (IgG1), TEPC 15 (IgA) and MOPC 315 (IgA) on protein A-Sepharose. The column was eluted with PBSA (0.12 M NaCl, 0.03 M phosphate, 0.02% sodium azide, pH 7.2) followed by 0.1 M glycine-HCl, pH 3.0. The buffers contained 0.1% human serum albumin. Fractions containing 0.5 ml were collected and the radioactivity measured in a gamma counter.

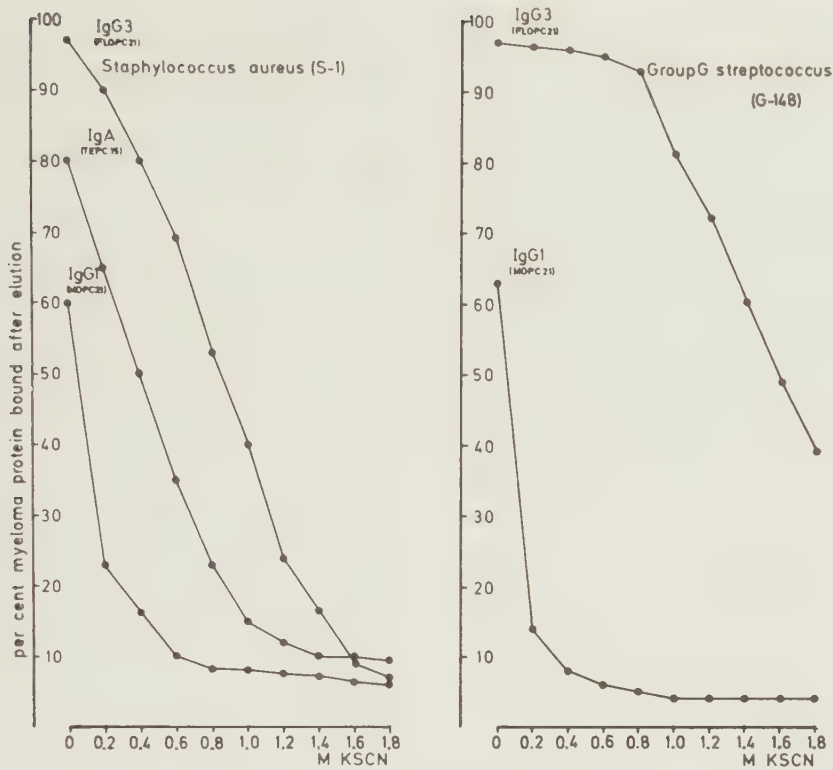


FIG. 5. Elution of radiolabelled myeloma components MOPC 21 (IgG1), FLOPC 21 (IgG3) and TEPC 15 (IgA) bound to *Staphylococcus aureus* strain (S-1) and to a group G streptococcus (G-148). The amount of radioactivity remaining on the bacteria after the elution steps is plotted as the percentage of the radioactivity initially bound to the pellet. TEPC 15 did not bind to the group G strain and is therefore not plotted.

TABLE I. Binding of radiolabelled murine myeloma proteins to protein A-Sepharose

Myeloma protein	Percentage of applied sample eluted with:	
	PBSA*	Glycine-HCl
MOPC 21 (IgG1)	6	94
TEPC 15 (IgA)	28	72
MOPC 315 (IgA)	96	4

* PBSA = 0.12 M NaCl, 0.03 M phosphate, 0.02% sodium azide, pH 7.2.

off at low molarities (Fig. 5). After the bacteria were suspended in PBSA (0.12 M NaCl, 0.03 M phosphate, 0.02% sodium azide, pH 7.2) only

60% was still bound to the bacterial pellet, and a 0.6 M KSCN solution reduced the bound fraction to about 10%. The binding of TEPC 15 (IgA) to S-1 was more stable, requiring KSCN solutions of higher molarities to dissociate the immunoglobulin (Fig. 5). A 1.4 M solution was needed for a reduction to 10%. FLOPC 21 (IgG3) presented the most stable binding. Suspension in PBSA had no effect on the binding. A difference was observed in the stability of FLOPC 21 bound to S-1 and to G-148. The binding to S-1 was less stable than the G-148 binding. A 1.6 M KSCN solution reduced the radioactivity bound to S-1 to 10%, in contrast to the 50% reduction seen with G-148. MOPC 213 (IgG2a) and MOPC 141 (IgG2b) presented a dissociation pattern similar to that of FLOPC 21.

DISCUSSION

Monoclonal immunoglobulins secreted by plasma cell tumour lines in inbred mice have permitted the definition of major classes of murine serum immunoglobulins [21]. Four IgG subclasses are recognized [21]. The present studies demonstrate binding of all four murine IgG subclasses to *S. aureus* strains (Fc-binding surface structure type I) (Table II). The various myeloma proteins were tested with the same bacterial suspension under identical conditions. The observed levels of uptake in the binding assay are therefore a reflection of avidity, since equilibrium existed between free and bound immunoglobulin. Thus the high levels of binding of IgG subclasses 2a, 2b and 3 reflect a strong binding to staphylococci in contrast to a much weaker binding of IgG1. The dissociation experiments performed with isothiocyanate confirmed these differences. The binding of IgG1 to whole staphylococci was more easily dissociated than the binding of the three other subclasses. These results are in agreement with recent studies performed by other investigators using affinity chromatography on protein A-Sepharose [17].

One of three tested murine IgA myeloma proteins, TEPC 15, showed a definite binding

to *S. aureus* (Table II). The specificity of this interaction was confirmed by affinity chromatography on protein A-Sepharose and by inhibition studies using polyclonal human IgG. Chromatography on glyceryl-coated porous glass beads revealed that the preparation contained not only monomeric but also aggregated myeloma protein. Binding studies performed on collected fractions showed that only the aggregated form was bound to protein-A-carrying staphylococci. Thus the physical state of immunoglobulins may be of critical importance for the possible expression of sites with affinity for bacterial surface structures. This observation deserves further attention, since it might form a basis for methods to detect aggregated immunoglobulins.

Binding of human IgA myeloma proteins to *S. aureus* is subclass-specific [8, 22]. Subclass IgA2 is protein-A-reactive, in contrast to IgA1. Human IgM myeloma proteins also form two similar groups differing in protein A reactivity [8, 15]. These groups are considered to represent human IgM subclasses. One of three murine IgA myeloma proteins included in the present investigation showed protein A reactivity. In a study of murine immunoglobulins Mackenzie *et al.* [16] demonstrated binding of one of five monoclonal myeloma proteins of IgM class

TABLE II. Binding of radiolabelled murine myeloma proteins to Gram-positive cocci*

Immunoglobulin		Fc binding type I, <i>S. aureus</i> protein A	Fc binding type II, group A streptococci	Fc binding type III, streptococci	
				Group C	Group G
IgG1	MOPC 21	+	—	—	—†
IgG2a	UPC 10	+++	—	++	++
	MOPC 213	+++	—	++	++
	PC 5	+++	—	++	++
IgG2b	MOPC 195	+++	—	++	++
	MOPC 141	+++	—‡	++	++
IgG3	J 606	+++	—	++	++
	FLOPC 21	+++	—	+++	+++
IgA	MOPC 315	—	—	—	—
	S 122	—	(++)§	—	—
	TEPC 15	—	—	—	—
IgM	MOPC 104 E	—	—	—	—

* Positive signs, +, ++, and +++, denote low, medium, and high levels of binding.

† Four of fifteen tested showed a weak interaction.

‡ One single strain, A-69, showed a low uptake that could be inhibited by human IgG.

§ The binding could not be inhibited by human IgG.

These studies suggest a situation similar to that observed in humans. Thus the difference in protein A reactivity could reflect the existence of murine IgA and IgM subclasses.

Group C and G streptococci (Fc-binding surface structure type III) bind murine IgG subclasses 2a, 2b and 3 (Table II). The observed differences in binding levels of individual strains demonstrate a great variability with a continuous spectrum of binding capacities. Individual strains did not show any preferential binding of any subclass. Highly reactive group G strains were, however, capable of binding not only IgG subclasses 2a, 2b and 3 but also small amounts of IgG1. The low levels of uptake and the low stability in dilute isothiocyanate solutions indicate that IgG1 is weakly bound, in contrast to the stronger binding of the three other subclasses. The ability of polyclonal human IgG to inhibit the uptake of murine myeloma components belonging to all four subclasses indicates that a single surface component is responsible for the binding of both murine and human immunoglobulins.

IgA myeloma protein S 122 bound exclusively to group A streptococci. This reactivity could not be inhibited by polyclonal human IgG. This binding therefore does not represent an ordinary Fc-mediated reactivity. The interaction noted might be due to Fab-mediated antibody binding. As yet, no antigen specificity is known for this monoclonal protein. Another alternative is a different type of non-immune immunoglobulin receptor present on group A streptococci. Another atypical binding was noted between the IgG2b myeloma protein MOPC 141 and the group A streptococcus A-69. This interaction could be inhibited by normal human IgG, which suggests the presence of a surface receptor with Fc-binding properties. Studies using animal sera for definition of the inhibition profile might indicate whether this strain carries a true type II receptor or an aberrant one similar to type I or III.

Group A streptococci (Fc-binding surface structure type II) were incapable of binding murine IgG except for a single strain that bound 20% of an IgG2b myeloma component (Table II). This almost total lack of reactivity with murine IgG contrasts with the positive binding of human IgG to most group A strains [19]. In comparison with the broad reactivity of staphylococcal protein A with immunoglobulin from

most mammalian species, the difference in affinity between human and mouse immunoglobulins for group A streptococci provides an alternative Fc binding reagent with interesting possible applications. One of the drawbacks of protein-A-carrying staphylococci in radioimmunoassays in identification procedures using specific antibodies is the simultaneous binding of immunoglobulins present in the test sample. In combination with specific rabbit antibodies with Fc reactivity for group A streptococci, such bacteria can be used in mouse models without interference of non-immune mouse immunoglobulin binding, for instance in cell surface antigen analysis.

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Demonstration of a New Type of Immunoglobulin G Receptor in *Streptococcus zooepidemicus* Strains

ERLING B. MYHRE* AND GÖRAN KRONVALL

Department of Medical Microbiology, University of Lund, S-22363 Lund, Sweden

Forty-seven bacterial strains representing four different group C streptococcal species were tested for binding of human and bovine immunoglobulin G (IgG) subclasses. Specific binding sites for IgG were found in all bacterial species studied. The four species included differed, however, in their capacities to interact with various IgG subclasses, indicating the existence of different types of IgG receptors. *Streptococcus equisimilis* and *Streptococcus dysgalactiae* were found to carry the same type of IgG receptor, one that is identical to the previously described Fc-binding structure type III. A new type of bacterial IgG receptor was detected in *Streptococcus zooepidemicus* strains. This receptor exhibits a protein A-like subclass specificity, with binding of human IgG1, IgG2, and IgG4 and of bovine IgG2. However, differences in their capacities to interact with other non-human immunoglobulins indicated that these two immunoglobulin-reactive structures were different. All types of IgG receptors in group C streptococci were found to be heat stable but susceptible to proteolytic enzymes. The inability of human serum albumin or fibrinogen to inhibit the uptake of radiolabeled IgG shows that the IgG receptor is separate from binding sites for these two other proteins on the bacterial cell surface. The existence of similar IgG receptors in closely related streptococcal species suggests that these structures have a common origin.

Staphylococcus aureus strains and most group A, C, and G streptococci possess surface structures with high affinity for the Fc portion of the immunoglobulin molecule (6, 12-14, 21). Previous studies have shown that such nonimmune reactivity is mediated by three major types of immunoglobulin G (IgG-Fc) receptors: type I, represented by staphylococcal protein A; type II, carried by group A streptococci; and type III, common to beta-hemolytic human group C and G streptococci (21, 22). Both streptococcal immunoglobulin receptors can interact with all four human IgG subclasses, in contrast to the restricted specificity of protein A (3, 18).

We have recently extended our initial studies to include streptococcal strains of non-human origin. Binding studies performed with human and bovine IgG revealed that bovine group G streptococci carried Fc receptors different from type III found in human group G strains (20). This observation prompted similar studies of four different group C streptococcal species. The present investigation was designed to determine the capacity of a representative collection of group C streptococci to bind human and bovine IgG subclasses. The capacity of other mammalian gamma globulins to interact with the IgG receptor was also explored by performing inhibition experiments with pooled animal sera. The present series of experiments describe a new

type of streptococcal IgG receptor found exclusively in *Streptococcus zooepidemicus* strains. This receptor is characterized by a subclass specificity similar to that of protein A, but it differs from protein A with respect to reactivity with animal gamma globulins.

MATERIALS AND METHODS

Bacterial strains. A total of 47 group C streptococci representing four different group C bacterial species were included in the studies (Table 1). Ten *Streptococcus equisimilis* strains and two *S. zooepidemicus* strains were obtained from human specimens received at the Clinical Microbiology Laboratory, University Hospital, Lund, Sweden. The remaining 35 non-human strains (*S. zooepidemicus*, 16; *Streptococcus dysgalactiae*, 12; and *Streptococcus equi*, 7) were kindly provided by O. Holmberg, State Veterinary Laboratory, Stockholm, Sweden; M. Wierup, Veterinary School, Uppsala, Sweden; and E. Kjems, State Serum Institute, Copenhagen, Denmark. Lancefield grouping was performed with a coagglutination method (2). Species identification was based on carbohydrate fermentation tests and on the type of hemolysis observed when strains were grown on horse blood agar plates (5). A *Streptococcus agalactiae* strain (B-1) was included as an IgG (Fc)-negative control strain.

Immunoglobulin preparations. Human serum samples containing monoclonal myeloma components of high concentrations and reduced background IgG were separated by zone electrophoresis in 0.6% aga-

TABLE 1. Percent binding of radiolabeled human and bovine IgG immunoglobulin preparations to different group C streptococcal species^a

Immunoglobulin	Binding capacity (%) to strains of:			
	<i>S. equisimilis</i> (n = 10)	<i>S. dysgalactiae</i> (n = 12)	<i>S. zooepidemicus</i> (n = 18)	<i>S. equii</i> (n = 7)
Human IgG1				
Per Gm (4) λ	79 (74-84)	66 (8-83)	28 (10-71)	23 (21-25)
And Gm (1) κ	90 (65-93)	77 (5-95)	37 (5-91)	17 (13-24)
Human IgG2				
Car κ	79 (72-84)	65 (6-92)	20 (2-71)	9 (6-10)
Sve λ	67 (60-80)	53 (4-75)	17 (3-49)	9 (8-10)
Human IgG3				
Blo Gm (5) κ	87 (66-90)	66 (7-89)	4 (2-6)	5 (3-6)
Arv Gm (5) κ	73 (60-73)	57 (6-78)	6 (3-11)	5 (3-7)
Fis Gm (5) λ	47 (42-53)	27 (4-56)	5 (4-8)	4 (2-6)
Lin Gm (5) κ	40 (35-44)	29 (3-43)	2 (2-3)	3 (2-5)
Vil Gm (5) λ	72 (66-77)	42 (4-63)	9 (8-10)	9 (8-10)
Wel Gm (21) κ	67 (53-71)	33 (2-78)	4 (2-9)	5 (2-10)
Human IgG4				
Far λ	77 (73-81)	61 (2-81)	31 (3-65)	20 (17-21)
Ben κ	69 (59-81)	63 (6-77)	38 (7-67)	32 (30-31)
Bovine				
IgG1 polyclonal	82 (75-88)	41 (4-77)	10 (7-14)	8 (6-9)
IgG2 polyclonal	92 (86-95)	73 (13-95)	28 (15-43)	22 (15-37)

^a Binding capacities are shown as arithmetic mean values of uptake levels of individual strains and as ranges of immunoglobulin binding.

rose, using 0.0075 M Veronal buffer (9). Isolated myeloma protein components were then subjected to gel filtration on a G-200 Sephadex column (2.5 by 96 cm) eluted at 4°C with phosphate-buffered saline (PBSA; 0.12 M NaCl, 0.03 M phosphate, 0.02% sodium azide, pH 7.2). Fractions containing monomeric IgG were pooled and used in all experiments. Proteolytic fragmentation during storage at 4°C was minimized by the addition of the plasmin inhibitor ε-aminocaproic acid (Epsikapron, AB Kabi, Stockholm, Sweden) to a final concentration of 3% (wt/vol). Immunoelectrophoresis of purified immunoglobulin preparations showed single precipitation lines when tested at a concentration of 1 mg/ml against rabbit anti-human IgG and anti-human serum proteins (Dako A/S, Copenhagen, Denmark). IgG subclass determination of the human myeloma proteins was kindly performed by V.-A. Oxelius, using a gel precipitation technique with rabbit antisera (24). Genetic marker (Gm) typing was kindly performed by R. Grubb (7). Human IgG was quantified by spectrophotometry by using an absorbance value of 13.5 at 280 nm. Purified polyclonal bovine IgG1, IgG2, and rabbit anti-bovine IgG and anti-bovine serum proteins were purchased from Miles Laboratories Inc., Elkhart, Ind.

Radiolabeling of immunoglobulins. Purified immunoglobulin preparations were labeled with ¹²⁵I (code no. IMS 30, Radiochemical Centre, Amersham, England) by mild electrolysis, as described by Rosa et al. (25), and modified for radioiodination of immunoglobulins (8). Incorporation of ¹²⁵I into protein was estimated by paper chromatography (8). Free, noncovalently bound isotope was removed by extensive dialysis against PBSA containing 0.05% Tween 20 (PBSA-Tween). Labeled preparations were then gel filtered on a G-200 Sephadex column. Monomeric

IgG's were used in all experiments. Labeled preparations showed specific activities of approximately 1 mCi/mg of protein. Purity was checked by performing immunoelectrophoresis of labeled immunoglobulin preparations mixed with normal serum and with polyclonal IgG. Autoradiography of the immunoelectrophoresis slides showed that the radioactivity was associated only with the IgG precipitation line.

Immunoglobulin-binding assay. Overnight Todd-Hewitt broth cultures of bacterial strains were washed twice and suspended in PBSA-Tween buffer to 10⁹ bacteria per ml, as calculated from optical density values (520 nm) of diluted samples in a photometer (Beckman model CP-1) (19). Binding studies were carried out at room temperature in triplicate in polystyrene test tubes (12 by 70 mm; Cerbo, Trollhättan, Sweden) by mixing 0.4 µg of radiolabeled immunoglobulin with 2 × 10⁸ bacterial organisms in a final volume of 225 µl. After 1 h, 2 ml of cold PBSA-Tween buffer was added and the bacteria were deposited by centrifugation at 4°C and 1,800 × g for 10 min. The supernatants were removed, and the radioactivity of the bacterial pellets was measured in a gamma counter (LKB Rack Gamma, model 1270, Biotec, Stockholm, Sweden). The immunoglobulin uptake was expressed as percentage of total radioactivity added. The reproducibility of the assay system was determined by testing 10 broth cultures of the human strain C-42 (*S. equisimilis*) for binding of human IgG1 (myeloma protein And). The binding levels (arithmetic mean of triplicate samples) showed that determinations could be performed with an imprecision of 5.5% (coefficient of variation). Binding to test tubes in the absence of bacteria was less than 1%. An Fc-negative control strain (B-1) showed uptake levels of 4 to 8%.

Estimation of immunoglobulin-binding capacity.

ity. Binding studies were performed with 1, 2, 3, 4, 5, 10, 15, 20, 25, and 30 μg of radiolabeled monomeric human IgG1 (myeloma protein And) and heat-killed bacteria (2×10^8 organisms as counted in a Petroff-Hauser chamber) in a final volume of 500 μl of PBSA-Tween buffer. Nonspecific binding was estimated by including a sample containing 1 mg of unlabeled myeloma protein. Specific binding was then calculated by subtracting from the radioactive uptake the amount of radioactivity that could not be displaced by unlabeled immunoglobulin.

Inhibition experiments. Representative strains of *S. equisimilis* (C-42), *S. zooepidemicus* (FS-3), and *S. dysgalactiae* (OH-13) were cultured for 16 h in 2 liters of Todd-Hewitt broth. Harvested bacteria were washed and suspended in 200 ml of PBSA and then conserved by heating the suspensions to 80°C for 5 min in a water bath. Bacteria were again washed and suspended to a concentration of 10^9 organisms per ml as determined by bacterial counts performed in a Petroff-Hauser chamber. Heat treatment as described did not affect the immunoglobulin reactivity.

(i) **Human IgG.** Polyclonal human IgG (AB Kabi) was gel filtered on a G-200 Sephadex column, and fractions containing monomeric IgG were used to inhibit the uptake of radiolabeled human IgG1 (myeloma protein And) to 2×10^8 test bacteria. Amounts ranging from 0.2 to 500 μg of IgG were mixed with 0.4 μg of radiolabeled IgG1, and the uptake to heat-treated bacteria was determined in a final volume of 500 μl .

(ii) **Human serum albumin and fibrinogen.** Inhibition experiments were performed as described above with purified serum albumin (23) and fibrinogen (16). The strain used in these experiments (C-42) has receptors for both of these proteins.

(iii) **Animal serum samples.** Inhibition experiments were also performed with serum samples from the following mammalian species: human, rabbit, rat, guinea pig, dog, cat, horse, pig, sheep, and cow.

Proteolytic digestion. Increasing amounts of pepsin and trypsin were added to 10^9 heat-treated bacteria suspended in 1.0 ml of 0.1 M acetate buffer (pH 4.5) and in 1.0 ml of 0.15 M phosphate buffer (pH 7.5), respectively. To prevent sedimentation of the bacteria during digestion, test tubes were rotated slowly. After 1 h of digestion at 37°C, the reaction was terminated by adding 100 μl of 1 M tris(hydroxymethyl)aminomethane base to the peptic digest and 1 ml of trypsin inhibitor solution (1 mg/ml in PBSA) to trypsinized bacteria. The bacteria were washed and resuspended in PBSA-Tween buffer and then tested for binding of radiolabeled human IgG1 (myeloma protein And). Pepsin, trypsin, and trypsin inhibitor were purchased from Sigma Chemical Co., St. Louis, Mo. (catalog no. P 7012, T 8553, and T 9128).

RESULTS

Quantitative binding of immunoglobulins. Forty-seven bacterial strains representing four different group C streptococcal species were tested for binding of radiolabeled human and bovine immunoglobulins. Twelve human IgG myeloma proteins and two bovine IgG prepara-

tions were included (Table 1). This immunoglobulin collection includes all human and bovine IgG subclasses and represents the most common human allotypes.

The 10 human beta-hemolytic *S. equisimilis* strains studied were all similar in their reactivity, with positive binding of all human and bovine immunoglobulin preparations (Table 1). High levels of binding (67 to 90%) were observed with all IgG1, IgG2, and IgG4 myeloma proteins and with four IgG3 preparations. Two IgG3 immunoglobulins (monoclonal proteins Lin and Fis) were less reactive, with mean binding capacities of 40 and 47%, respectively. These two myeloma proteins were both genetic marker type 5 [Gm(5)] but differed in the type of light chain. The interaction with bovine IgG was characterized by a higher uptake of IgG2 than of IgG1.

Twelve alpha-hemolytic bovine *S. dysgalactiae* were included (Table 1). Nine of these isolates were reactive, with mean uptake levels ranging from 27 to 77% for human immunoglobulins. The lowest reactivity was again observed with the human IgG3 myeloma proteins Lin and Fis. A difference in reactivity with bovine IgG subclasses was noted. Individual *S. dysgalactiae* strains capable of immunoglobulin binding showed nearly twofold higher uptake of bovine IgG2 than of IgG1.

A distinct pattern of immunoglobulin reactivity was observed with nine bovine and two human beta-hemolytic *S. zooepidemicus* strains. Positive binding was detected with all six human IgG1, IgG2, and IgG4 myeloma proteins (Table 1). In contrast, all six IgG3 immunoglobulin preparations were nonreactive. Individual *S. zooepidemicus* isolates varied in degree of reactivity, as reflected in the binding levels, which ranged from 20 to 91% for human IgG subclasses 1, 2, and 4. Strains showing high levels of uptake with one myeloma protein were also the most potent binders of the other reactive preparations. A low but definite reactivity was observed with bovine IgG2 in contrast to the nonreactive IgG1 subclass. Nine non-human *S. zooepidemicus* strains were incapable of binding any of the immunoglobulins included in the investigation.

Seven equine *S. equii* strains formed a homogeneous group, binding small quantities of human IgG1 and IgG4 myeloma proteins. In contrast, the uptake of monoclonal human IgG2 and IgG3 proteins did not exceed that recorded with the Fc-negative *S. agalactiae* strain. The reactivity with bovine immunoglobulins was similar to that of *S. zooepidemicus* strains, with binding of only the IgG2 subclass.

Representative strains of *S. equisimilis* (C-42), *S. dysgalactiae* (OH-13), and *S. zooepidem-*

icus (FS3) were selected for further studies. These strains were selected for the high degree of reactivity observed in the initial binding studies. The immunoglobulin-binding profile of these strains is shown in Fig. 1. The uniform reactivity of C-42 and OH-13 with all human IgG subclasses and the restricted specificity of FS-3 are clearly seen. The maximal binding capacity was determined by testing increasing amounts of a radiolabeled human IgG1 myeloma protein (And) (Fig. 2). Strain FS-3 showed the highest capacity, binding more than 5.5 μ g of human IgG. The corresponding quantities bound by C-42 and OH-13 were 4.0 and 2.9 μ g of IgG, respectively. For comparison, *S. aureus* strain Cowan I was also included and showed a maximal binding level of 12 μ g. The nonspecific binding recorded with the Fc-negative control strain was 0.6 μ g of IgG.

The specificity of the IgG receptor was explored by performing inhibition experiments with unlabeled polyclonal human IgG, human serum albumin, and fibrinogen, proteins which are capable of interaction with streptococci. Polyclonal IgG inhibited the binding of isotope-labeled myeloma protein to all three test strains (Fig. 3). The IgG quantity giving a defined degree of inhibition was, however, different. A 50% inhibition of the uptake to strains FS-3, C-42, and OH-13 was seen after the addition of 12, 6, and 4 μ g, respectively, of inhibitory protein. The slopes of the three inhibition curves were quite similar. Addition of up to 500 μ g of human serum albumin and fibrinogen to the test system had no effect on the IgG binding. These amounts were sufficient to saturate the albumin and the fibrinogen receptors present on the test organisms.

Binding of non-human immunoglobulins. The capacity of the three group C streptococcal species to interact with non-human immunoglobulins was determined in inhibition experiments that used serum pools from nine mam-

malian species. Aliquots (10 μ l) of animal serum samples were mixed with isotope-labeled human IgG myeloma protein, and the uptake of radioactivity to selected test strains was measured (Fig. 4). Serum samples from rabbits, guinea pigs, horses, pigs, sheep, and cows tested with the *S. equisimilis* strain (C-42) were inhibitory (Fig. 4A). In contrast, addition of sera from rats, dogs, and cats resulted in little or no inhibition. Similar results were obtained when the *S. dysgalactiae* strain (OH-13) was studied (Fig. 4B). A different inhibition pattern was observed with the *S. zooepidemicus* strain (FS-3) (Fig. 4C). Only serum samples from rabbits, guinea pigs, and pigs showed inhibition. Serum samples from horses, sheep, and cows were nonreactive with FS-3.

Four additional strains of each of the three group C streptococcal species studied were also tested in inhibition experiments with the same panel of animal sera. The inhibition profile of the individual strains was equivalent to that of the appropriate type strain. Thus, sera from

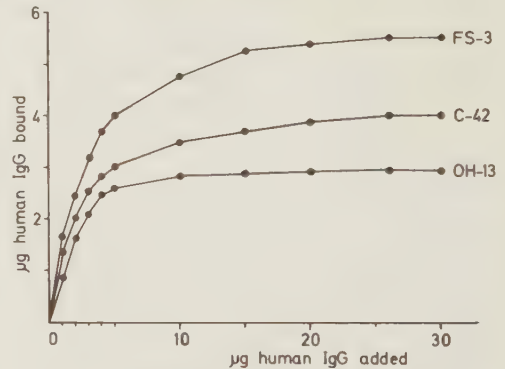


FIG. 2. Uptake of radiolabeled monoclonal human IgG1 to 2×10^8 organisms of selected group C strains tested with 1- to 30- μ g quantities of myeloma protein And in a final volume of 500 μ l.

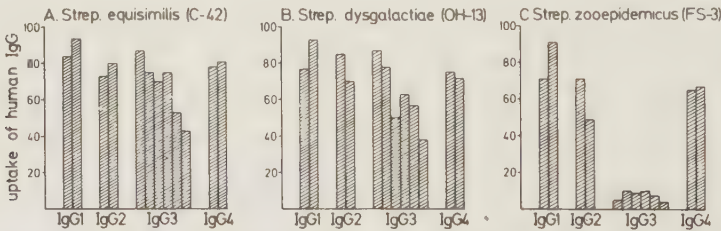


FIG. 1. Binding of 12 human IgG myeloma proteins to selected bacterial strains belonging to three different group C streptococcal species. Strains C-42, OH-13, and FS-3 represent the species *S. equisimilis*, *S. dysgalactiae*, and *S. zooepidemicus*. The uptake of 0.4 μ g of isotope-labeled immunoglobulin to 2×10^8 bacterial organisms is expressed as percentage of added radioactivity.

horses, cows, and sheep differed most markedly in inhibiting capacity. The capacity of 10- μ l quantities of these three sera to differentiate between the various types of IgG receptors in group C streptococci was further explored in inhibition experiments with increasing amounts of serum (Fig. 5). The previously described similarity between strains C-42 and OH-13 was confirmed. Positive inhibition was obtained with horse, cow, and sheep sera (Fig. 5A and B). Small quantities of cow and sheep sera were, however, more inhibitory with OH-13 than with C-42. This tendency is even more distinct when the lower maximal binding capacity of the former strain is taken into account. Thus, the IgG receptor of OH-13 seems to have a higher affinity for bovine and ovine immunoglobulins than does the corresponding structure of C-42. Cow and

sheep sera added in quantities up to 100 μ l had no effect on the uptake of labeled human IgG to FS-3 (Fig. 5C). In contrast, horse serum showed a dose-related inhibition.

Proteolytic digestion. Bacterial suspensions of the selected type strains were digested with increasing amounts of trypsin and pepsin and then tested for uptake of isotope-labeled human IgG (Fig. 6). Exposure to trypsin and pepsin resulted in a reduction in immunoglobulin-binding capacity, indicating that the immunoglobulin receptors are susceptible to both enzymes. However, characteristic differences in sensitivity were noted among the test strains. FS-3 (*S. zooepidemicus*) was exquisitely sensitive to trypsin, in contrast to the more stable structures of C-42 (*S. equisimilis*) and OH-13 (*S. dysgalactiae*) (Fig. 6A). Pepsin digestion had a slightly different effect (Fig. 6B). As little as 10 μ g of pepsin abolished the immunoglobulin reactivity of FS-3 and OH-13. A similar reduction in reactivity of C-42 was seen after addition of 500 to 1,000 μ g of pepsin. The sensitivities of 16 other group C strains were also determined in similar studies with bacteria digested with 20- and 500- μ g quantities of trypsin and pepsin. These experiments gave results quite similar to those reported for the selected type strains.

DISCUSSION

Group C streptococci can be classified into four well-defined species: *S. equisimilis*, *S. dysgalactiae*, *S. zooepidemicus*, and *S. equii* (5). The present studies demonstrate that these four bacterial species can all bind human immunoglobulin in a nonimmune way. The high levels of uptake of several human monoclonal myeloma proteins and the inhibiting capacity of polyclonal human IgG suggest that the reactivity resides in the constant portion of the immunoglobulin molecule. Differences in the binding

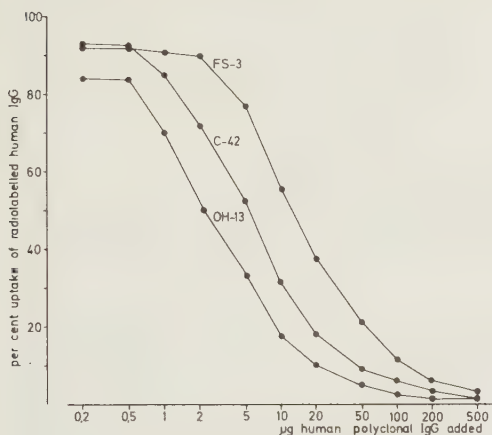


FIG. 3. Capacity of human polyclonal IgG to inhibit the uptake of labeled human IgG to selected group C strains. Increasing amount (0.2 to 500 μ g) of unlabeled immunoglobulin were mixed with 0.4 μ g of myeloma protein And, and the uptake was determined.

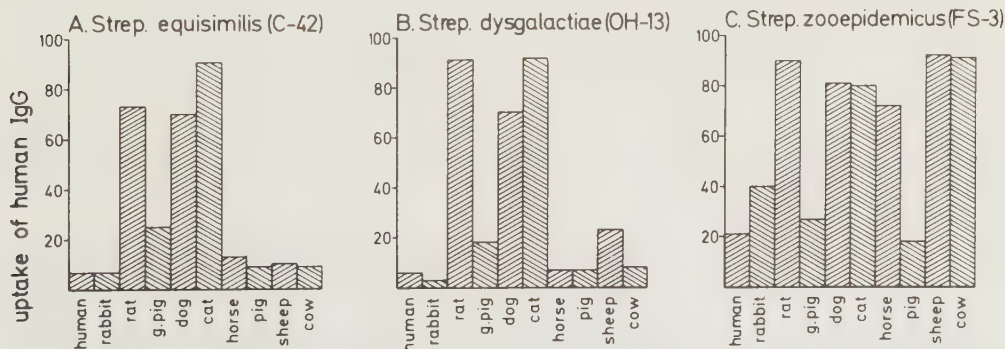


FIG. 4. Capacity of mammalian immunoglobulin to compete with isotope-labeled human IgG for binding to selected group C strains. A 10- μ l amount of serum was mixed with labeled human IgG, and the binding to 2×10^8 bacteria was determined. g. pig, Guinea pig. Uptake is expressed as percentage of added radioactivity.

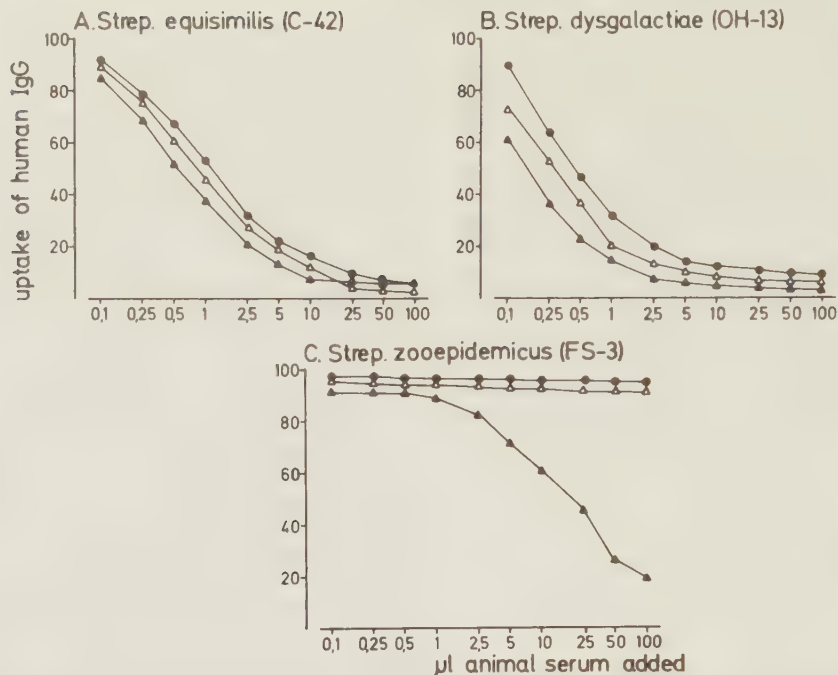


FIG. 5. Capacity of increasing amounts 0.1- to 100- μ l quantities of horse (▲), cow (△), and sheep (●) sera to inhibit the binding of labeled human IgG to selected strains. Uptake is expressed as percentage of added radioactivity.

of human and bovine IgG subclasses reflect the existence of different variants of IgG receptors in group C streptococci. The patterns observed could be correlated to defined species (Table 2). The IgG receptor of *S. equisimilis*, which is identical to the previously described Fc-binding structure type III, is also carried by *S. dysgalactiae* strains. This receptor is characterized by a broad reactivity which includes all human and bovine IgG subclasses. The immunoglobulin reactivity found in *S. equi* was less significant, with binding levels slightly higher than those obtained with an Fc-negative control strain. A new type of streptococcal IgG receptor was detected in *S. zooepidemicus* strains. This structure has a subclass specificity similar to that of staphylococcal protein A, with binding of human IgG1, IgG2, and IgG4 and bovine IgG2. The two immunoglobulin-reactive components could, however, be differentiated in inhibition experiments employing a panel of animal sera. Whereas cat and dog sera showed minimal inhibitory effects in the present experiments (Fig. 4), both sera have been found to be highly inhibitory in previous studies of protein A-carrying staphylococci (21). More direct evidence has recently been obtained with isotope-labeled ca-

nine IgG. The preparation gave no uptake to *S. zooepidemicus* strains, in contrast to a positive binding to *S. aureus* isolates (unpublished observations). Thus, the new streptococcal IgG receptor on *S. zooepidemicus* strains and protein A are different, but closely related, types of Fc receptors.

The interaction with non-human immunoglobulins was explored by performing inhibition experiments with a panel of animal sera. Our initial definition of the major types of IgG receptors in gram-positive cocci was based on such experiments. The validity of this approach was also confirmed in the present investigations. The three different inhibition patterns observed could be correlated to the corresponding type of IgG receptor as defined by the reactivity with human and bovine IgG subclasses. These new inhibition profiles and those of *S. aureus* and group A streptococci are shown in Table 3.

The surface component responsible for the binding of immunoglobulin to bacteria has not yet been identified, but some characteristic features were observed. Heat treatment of bacteria at 80°C for 5 min did not affect their IgG-binding capacity. Digestion with proteolytic enzymes, however, resulted in reduced binding activity.

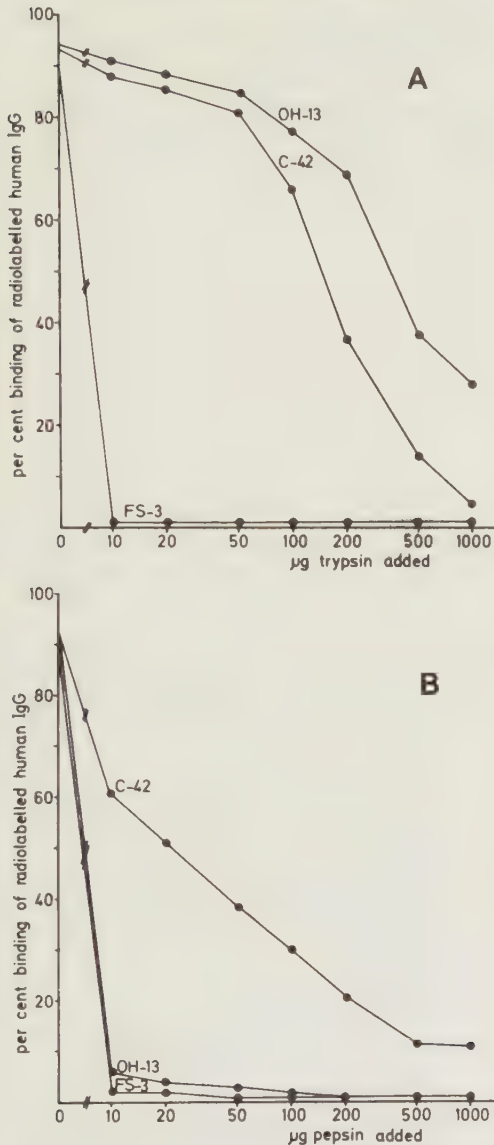


FIG. 6. Effect of trypsin (A) and pepsin (B) on heat-treated bacteria tested for uptake of labeled human IgG1. Increasing quantities of enzyme were used for digestion of 10^8 bacteria at 37°C for 1 h.

Thus, the two types of IgG receptors found in group C streptococci appear to be heat-stable surface components which are probably of a protein nature or may be connected to the bacterial surface by a protein structure. The inability of human serum albumin and fibrinogen to inhibit the immunoglobulin uptake indicates

TABLE 2. Binding of human and bovine IgG subclasses to selected, highly reactive strains of group C streptococci of various species

Immunoglobulin subclass	Binding level ^a		
	<i>S. equisimilis</i>	<i>S. dysgalactiae</i>	<i>S. zooepidemicus</i>
Human			
IgG1	++	++	++
IgG2	++	++	++
IgG3	++	++	—
IgG4	++	++	++
Bovine			
IgG1	++	++	—
IgG2	++	++	++

^a + and ++ denote low and high binding levels, respectively.

that the IgG receptor is separate from the binding sites for these two proteins. Several streptococcal strains have been maintained in our laboratory for more than 1 year by repeated subculturing without loss of IgG reactivity.

The present investigation provides some insight into the evolutionary history of streptococci. The existence of different types of IgG receptors in related streptococcal species can be explained by the occurrence of an original immunoglobulin-binding structure during the evolution before the separation of streptococci into different species. With the separation of streptococci into recognizable species this primitive structure evolved into the present types of IgG receptors. Thus, the observation that *S. equisimilis*, *S. dysgalactiae*, and beta-hemolytic human group G streptococci carry identical IgG receptors implies that they are more closely related to each other than to other streptococcal species and are of a closer evolutionary origin.

It is still unclear why some bacterial species have developed surface structures with a high affinity for immunoglobulins. Such structures might, however, be of significance for the host-parasite relationship. Acquisition of host-derived IgG could, for instance, enable the bacterial cell to escape recognition and normal disposal by the host. Such a mechanism is used by the mammalian parasite *Schistosoma mansoni* (1). This parasite has tegumental binding sites for blood group substances (4), histocompatibility antigens (26), and immunoglobulin (10, 27). Group C streptococci are also capable of several interactions with host proteins. Binding sites have been described for human serum albumin (17, 23), fibrinogen (16), haptoglobin (11), and aggregated β_2 -microglobulin (13, 15). This multitude of reactivities must be taken into account when the possible role of the IgG receptor in host-parasite relationships is considered.

TABLE 3. Capacity of animal serum pools to inhibit the uptake of radiolabeled human IgG to Fc-reactive, gram-positive cocci

Order	Species	Inhibition level ^a				
		<i>S. aureus</i> ^b	Group A streptococci ^b	<i>S. equis-milis</i>	<i>S. dysga-lactiae</i>	<i>S. zooepi-demicus</i>
<i>Lagomorpha</i>	Rabbit	++	++	++	++	++
	Rat	—	—	—	—	—
<i>Rodentia</i>	Guinea pig	++	—	++	++	++
	Dog	++	—	—	—	—
<i>Carnivora</i>	Cat	++	—	—	—	—
	Horse	—	—	++	++	—
<i>Perissodactyla</i>	Pig	++	++	++	++	++
<i>Artiodactyla</i>	Sheep	—	—	++	++	—
	Cow	—	—	++	++	—

^a ++, High levels of inhibition; —, low levels of inhibition.
^b Data from previous studies (21).

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IMMUNOCHEMICAL ASPECTS OF Fc-MEDIATED BINDING OF HUMAN IgG SUBCLASSES TO GROUP A, C AND G STREPTOCOCCI

ERLING B. MYHRE and GÖRAN KRONVALL

Department of Medical Microbiology, University of Lund, Lund, Sweden

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Abstract—Nineteen human IgG myeloma proteins were tested in a sensitive assay for binding to 60 strains of β -hemolytic streptococci. Human group A, C and G streptococci demonstrated strong reactivity with positive binding of all four human IgG subclasses. Group C and G streptococci were capable of binding twice as much IgG as group A strains. β -Hemolytic bovine group G streptococci showed a low degree of reactivity with IgG1 and IgG4. Kinetic experiments revealed that the IgG binding was a time-dependent, displaceable process. Studies performed with Fab and Fc fragments showed that Fc structures are involved in the binding of intact IgG molecules to the bacterial cell surface. In addition, radiolabelled Fab fragments from two monoclonal components exhibited a definite reactivity with group G streptococci. Scatchard plots of the IgG–streptococcal interaction showed a linear section at low saturation levels. The equilibrium constants derived from such plots were $8\text{--}9 \times 10^7$ l./mole. The equilibrium constant for the interaction of IgG with staphylococcal protein A as measured with the Cowan I reference strain was 5×10^7 l./mole.

INTRODUCTION

Non-immune binding of immunoglobulin G to *Staphylococcus aureus* is the result of an interaction between the Fc portion of the antibody molecule and protein A, a staphylococcal cell wall constituent (Forsgren & Sjöquist, 1966; Kronvall & Frommel, 1970). An analogous immunoglobulin reactivity has been found in group A, C and G streptococci (Kronvall, 1973). This reactivity is mediated by specific binding structures, Ig receptors, exposed on the surface of the streptococcal cell wall (Myhre & Kronvall, 1977). There are several distinct types of streptococcal IgG-Fc receptors (FcR). FcR type II is found in group A streptococci, FcR type III is present on human group C and G streptococci and FcR type IV is restricted to β -hemolytic bovine group G streptococci (Myhre & Kronvall, 1977; Myhre *et al.*, 1979). These immunoglobulin receptors differ from protein A (FcR type I) in terms of specificity for several non-human immunoglobulins (Myhre & Kronvall, 1977).

Protein A-reactive structures in the Fc fragment of human IgG can be detected in subclasses IgG1, IgG2 and IgG4 but not in IgG3 (Kronvall & Williams, 1969). Recent studies in our laboratory have shown that equine group C streptococci exhibit a protein A-like subclass specificity (Myhre & Kronvall, 1980c). Previous studies on human streptococci were performed

either using polyclonal human IgG or a limited number of monoclonal proteins (Christensen & Oxelius, 1974; Myhre & Kronvall, 1977). The aim of the present investigation was to explore more extensively the capacity of streptococci to bind human IgG subclasses. Experiments were also designed to delineate association and dissociation parameters of the receptor–IgG interaction and to determine the equilibrium constant and the number of IgG receptors on the bacterial cell.

MATERIALS AND METHODS

Human myeloma proteins

Serum containing high levels of monoclonal IgG was obtained from patients with multiple myeloma. Monoclonal proteins were isolated from serum samples by zone electrophoresis (Johansson, 1972) and purified by gel filtration on Sephadex G-200 (Myhre & Kronvall, 1980c). Purity was assessed by immunoelectrophoresis performed with rabbit antisera with specificity for human IgG, IgA, IgM, light chain κ , light chain λ and human serum proteins (Dako A/S, Copenhagen, Denmark). Isolated monoclonal proteins were all found to contain IgG without any other detectable protein. Protein A reactivity was tested by gel diffusion (Kronvall & Williams, 1969). IgG subclass determination was kindly performed by Dr. Vivi-Anne Oxelius using a gel

precipitation technique with rabbit antisera (Oxelius, 1978). Gm typing was kindly performed by Prof. R. Grubb (Grubb, 1970). IgG was quantified by spectrophotometry by using an adsorbance value ($E_{1\text{cm}}^{1\%}$) at 280 nm of 13.5.

Preparation of immunosorbent

Antibodies with specificity for human IgG Fab and Fc fragments were precipitated with 40% saturated ammonium sulphate from rabbit antiserum (Behring-Werke, Frankfurt, F. R. G.). The precipitate was dissolved in 0.2 M carbonate buffer, pH 8.5, and coupled to CnBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) as described by the supplier.

Fab and Fc fragments

Human polyclonal IgG (AB Kabi, Stockholm, Sweden), as well as three myeloma globulins, were digested with papain (Sigma Chemical Co, St. Louis, MO, U.S.A.) at an enzyme to substrate ratio of 1:100 (w/w) for 6 hr at 37°C and pH 7.0 (Michaelsen & Natvig, 1971). The digestion was terminated by addition of iodoacetamide to a final concentration of 0.01 M. The proteolytic fragments were separated from undigested IgG by gel filtration on a G-100 column (1.6 × 90 cm) equilibrated and eluted at 4°C with phosphate-buffered saline (PBSA, 0.12 M NaCl, 0.03 M phosphate, 0.02% sodium azide, pH 7.2). Fab and Fc fragments were isolated by affinity chromatography performed with anti-Fab and anti-Fc specific immunosorbent columns equilibrated with 0.1 M phosphate buffer, pH 7.0, containing 0.3 M NaCl. Glycine-HCl buffer (0.1 M, pH 3.0) was used for desorption of bound fragments.

Bacterial strains

A total of 80 bacterial strains were studied: 15 human group A streptococci, 15 human group C streptococci, 15 human group G streptococci, 15 bovine β -hemolytic streptococci, 15 *Staphylococcus aureus* strains and 5 group B streptococci (IgG-Fc negative control strains). The *S. aureus* strain Cowan I was included as a protein A positive reference strain.

IgG binding assay

Purified myeloma proteins were labelled with I^{125} (The Radiochemical Centre, Amersham, U.K.) by a modified electrolytic procedure (Myhre & Kronvall, 1980a). Assessment of labelled preparations by autoradiography

(Myhre & Kronvall, 1980c) showed that radioactivity was associated only with IgG. Binding studies were carried out as reported previously (Myhre & Kronvall, 1977). Briefly, triplicate samples containing 0.3 μg of radiolabelled myeloma protein were mixed with 2×10^8 bacteria in a final volume of 225 μl of PBSA containing 0.1% Tween 20 (PBSA-TWEEN). After 1 hr at room temperature 2 ml PBSA-TWEEN buffer was added and the bacteria deposited by centrifugation. The quantity bound to the bacterial pellet was expressed as per cent of added radioactivity. The non-specific uptake recorded with IgG negative group B streptococci was less than 5%.

Further studies were carried out with isotope-labelled IgG1 (Hed) and heat-killed (80°C for 5 min) bacteria. A control sample containing 1 mg of unlabelled IgG1 was included in all experiments. Corrections were made for this non-displaceable background binding. The effect of the number of test bacteria on the uptake was explored in an assay containing 0.3 μg of IgG1 and variable amounts of bacteria (5×10^6 – 2×10^9 organisms) in a final volume of 500 μl PBSA-TWEEN buffer. Group B streptococci were added to adjust the bacterial pellet to a final number of 2×10^9 bacteria.

The maximal binding capacity was determined by testing increasing quantities (2, 5, 10, 15, 20, 30, 40 and 50 μg) of IgG1 for binding to 5×10^8 bacteria.

Binding levels at different pH values were measured with bacteria suspended in 0.1 M citrate (pH 2.0–6.0) and phosphate (pH 6.5–8.0) buffers containing 0.1% Tween 20.

Inhibition assay

Increasing amounts of human polyclonal IgG, myeloma proteins, Fab and Fc fragments were mixed with 0.3 μg radiolabelled immunoglobulin in a final volume of 700 μl PBSA-TWEEN buffer, and the uptake to 2×10^8 bacteria was determined.

Kinetics studies

All experiments were performed in triplicate at room temperature with 0.3 μg labelled IgG1 myeloma protein and 2×10^8 heat-killed bacteria. Binding assays were performed with incubation times ranging from 10 sec to 60 min. Further uptake of labelled IgG was prevented by addition of 500 μl of ice-cold unlabelled human IgG (2 mg/ml in PBSA). For dissociation studies, bacteria were first reacted with isotope-labelled

immunoglobulin in a final volume of 50 μ l. After 1 hr the samples were diluted 70 times with PBSA-TWEEN buffer. After a further 5, 10, 20, 30, 45, 60, 120 and 360 min the amount still bound to the bacteria was determined. Rate constants were calculated as described by Cuatrecasas & Hollenberg (1976). The dissociation rate was computed by using the first order equation $K_2 = 2.303 (1/t) \log (x/x-a)$. In this equation a is the concentration of IgG, b is the concentration of receptor and x is the concentration of IgG-receptor complex at the time t .

Scatchard analysis

An IgG1 myeloma protein (Hed) was iodinated to a specific activity of 0.1 mCi/mg protein with a solid-phase lactoperoxidase method (David, 1972). Varying mixtures of labelled and unlabelled immunoglobulin were tested for reactivity without noticing any difference in binding capacity between the two components. The maximal uptake of labelled immunoglobulin to highly reactive bacteria was approximately 90%, indicating that 10% of the material was non-reactive. Binding studies were performed in triplicate by incubation of 2, 5, 10, 20, 30, 40 and 50 μ g quantities of labelled protein with 5×10^8 heat-killed bacteria in a final volume of 700 μ l of PBSA-TWEEN buffer. Bound and

free fractions were measured. After corrections for the non-reactive material the data were plotted as described by Scatchard (1949).

RESULTS

Binding of human IgG myeloma proteins

Nineteen human IgG myeloma proteins were tested in a sensitive assay for binding to 75 strains of Gram-positive cocci (Tables 1 and 2). This collection of myeloma proteins included all four IgG subclasses of the common allotypes. The bacterial strains represent species which carry previously defined types of IgG-Fc receptors (FcR) (Myhre & Kronvall, 1977; Myhre *et al.*, 1979).

Fourteen of 15 group A streptococci (FcR type II) showed positive binding of all myeloma proteins (Table 2). Individual strains varied greatly in reactivity as reflected in binding levels ranging from low to very high. Various myeloma proteins differed also in reactivity. When results obtained with individual strains of group A streptococci were pooled, uptake levels recorded with six IgG1 myeloma proteins ranged from 55 to 87%. The corresponding figures for IgG2, IgG3 and IgG4 were 42-62, 25-60 and 47-57%. No correlation was observed between the degree of binding and factors like Gm group, type of protein A reactivity and type of light chain.

Table 1. Characteristics of purified human IgG myeloma proteins tested for binding to Gram positive cocci

Myeloma	Allotype ^a	Light chain	Electrophoretic mobility	Protein A reactivity ^b	
IgG1	And	G1m(1)	κ	slow	P
	Hed	G1m(1)	κ	fast	I
	Ken	G1m(1)	λ	fast	P
	Gre	G1m(3)	λ	slow	I
	Ols.	G1m(3)	κ	stationary	P
	Per	G1m(3)	λ	stationary	I
IgG2	Sta	G2m(23)	κ	slow	P
	Hey	G2m(23)	κ	fast	I
	Car		κ	stationary	I
	Sve		λ	fast	I
IgG3	Wel	G3m(21)	κ	fast	
	Blo	G3m(5)	κ	stationary	
	Vil	G3m(5)	λ	fast	
	Fel	G3m(5)	κ	slow	
	Arv	G3m(5)	κ	slow	
	Lin	G3m(5)	λ	slow	
IgG4	Ben		κ	stationary	I
	Far		λ	fast	I
	Wer		κ	slow	P

^a Numeric designations (Ropartz *et al.*, 1976).

^b P and I denote precipitation and inhibition, respectively.

IgG3 myeloma proteins non-reactive.

Table 2. Binding of human IgG myeloma proteins to 68 bacterial strains representing 5 different immunoglobulin reactive species^a

Myeloma protein		Group A streptococci FcR II	Human group C streptococci FcR III	Human group G streptococci FcR III	Bovine group G streptococci FcR IV	Staphylococcus aureus FcR I
IgG1	And	71 (44-86)	93 (90-95)	93 (90-94)	44 (30-59)	87 (85-90)
	Hed	87 (79-92)	89 (85-92)	90 (88-93)	19 (14-21)	79 (76-84)
	Ken	72 (50-90)	79 (58-90)	77 (57-96)	15 (13-17)	89 (84-93)
	Gre	71 (47-89)	69 (51-91)	72 (51-95)	25 (22-29)	86 (75-90)
	Ols	55 (40-75)	86 (74-86)	89 (74-92)	32 (28-34)	75 (71-80)
	Per	57 (43-83)	92 (88-95)	91 (88-93)	24 (22-26)	81 (76-85)
IgG2	Sta	62 (45-81)	76 (42-80)	76 (65-85)	30 (26-33)	79 (73-83)
	Hcy	55 (21-84)	81 (62-83)	83 (75-92)	9 (6-8)	83 (75-88)
	Car	55 (31-79)	80 (77-84)	80 (79-91)	7 (6-8)	75 (75-83)
	Sve	42 (22-66)	65 (57-80)	64 (58-73)	7 (6-8)	69 (60-72)
IgG3	Wel	25 (9-44)	63 (36-73)	61 (43-79)	7 (6-8)	3 (2-4)
	Blo	48 (17-85)	89 (87-92)	89 (87-95)	3 (2-4)	4 (2-5)
	Vil	36 (17-66)	74 (68-77)	74 (68-77)	9 (8-10)	7 (4-8)
	Fel	40 (32-43)	50 (30-55)	48 (42-54)	3 (2-5)	5 (2-6)
	Arv	60 (40-70)	71 (57-75)	65 (35-71)	4 (4-5)	8 (7-9)
	Lin	44 (35-50)	54 (46-58)	58 (50-62)	3 (3-6)	3 (2-5)
IgG4	Ben	56 (39-72)	67 (37-81)	56 (47-78)	35 (32-42)	70 (68-71)
	Far	47 (30-63)	75 (59-81)	69 (25-83)	17 (14-18)	66 (59-70)
	Wer	57 (50-64)	58 (22-64)	59 (42-66)	49 (46-51)	62 (57-65)
No. positive strains		14	15	12	12	15
No. tested strains		15	15	15	15	15

^a Binding capacities are expressed as the per cent uptake of 0.3 µg of radiolabelled myeloma protein to 2×10^8 bacterial organisms. The results obtained with individual strains were pooled for each species and are shown as the arithmetic mean and as the range of binding levels.

Human group C and G streptococci (FcR type III) were more homogenous than group A streptococci in their reactivity. All 15 group C and 12 out of 15 group G strains showed immunoglobulin binding capacity (Table 2). The binding capacity for individual myeloma proteins varied from 50 to 90% (pooled results). Preparations which showed high uptakes to group A streptococci were also strongly bound by group C and G strains.

Twelve of 15 β -hemolytic bovine group G streptococci (FcR type IV) were positive. A distinct pattern of reactivity was observed with low levels of binding of all IgG1 and IgG4 proteins (Table 2). All six IgG3 preparations were non-reactive, demonstrating only background binding. The IgG2 preparation Sta showed a 30% uptake, in contrast to three other IgG2 myeloma proteins which were completely negative.

The protein A positive *Staphylococcus aureus* strains (FcR type I) formed a homogeneous group with high degrees of reactivity with IgG1, IgG2 and IgG4 (Table 2). Only background binding was noted with monoclonal components of IgG3 subclass. For a general comparison, the

immunoglobulin binding profiles of representative strains of group A (A-1), group C (C-42), human group G (G-148) and bovine group G (BG-6) streptococci are shown in Fig. 1 (A)-(D).

Subsequent experiments were performed with heat-killed bacteria (80°C for 5 min). This treatment did not affect immunoglobulin reactivity and heat-killed bacteria could be stored at 4°C as a suspension for at least 2 months without appreciable loss of reactivity.

Binding as a function of pH

Binding studies carried out with bacteria suspended in citrate and phosphate buffers with pH values ranging from 3.0 to 8.0 revealed that the binding of IgG1 was dependent on the hydrogen ion concentration (Fig. 2 A). At low pH values low levels of uptake was noted with all three strains. This tendency was more marked for strain A-1 than for G-148 and C-1 (Cowan I, *S. aureus* reference strain). All three strains showed maximal binding at pH values above 5.0.

Binding as a function of the number of bacteria

Binding levels were recorded with varying quantities of bacteria (5×10^5 – 2×10^9 or-

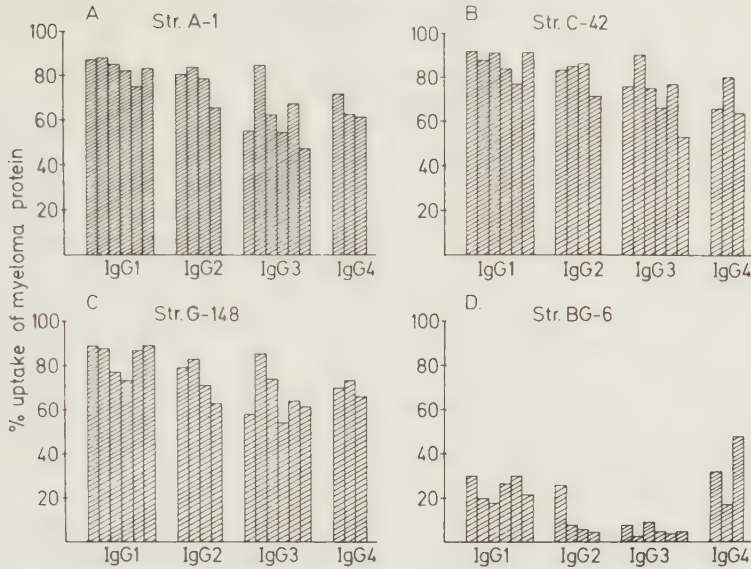


Fig. 1. Non-immune binding of 19 human IgG monoclonal proteins to selected strains representing group A (Str. A-1; Fig. 1 A), group C (Str. C-42; Fig. 1B), human group G (Str. G-148; Fig. 1 C) and bovine group G streptococci (Str. BG-6; Fig. 1 D). Three hundred nanogram isotope-labelled immunoglobulin were mixed with 2×10^8 bacterial organisms in 225 μ l 0.15 M phosphate buffered saline, pH 7.2, containing 0.02 M sodium azide and 0.1% Tween 20 (PBSA-TWEEN buffer). After 1 hr at room temperature 2 ml PBSA-TWEEN buffer were added. The bacteria were deposited by centrifugation and the radioactivity of the bacterial pellet determined. The IgG uptake is expressed as per cent of added radioactivity.

ganisms) in this assay. A steady increase in the bound fraction was observed with three selected test strains (Fig. 2 B). However, differences in binding capacity were noted. Larger quantities of the group A (A-1) and group G (G-148) strains than of *Staphylococcus aureus* (C-I) was required to achieve a defined uptake of IgG1 (Hed). Quantities exceeding 10^8 bacteria produced only small increments of binding, indicating a relative

excess of binding sites over labelled ligand in the test system. The non-specific binding was less than 5% (strain B-1).

Binding as a function of IgG concentration

Increasing amounts of IgG1 (Hed) were tested for binding to 5×10^8 bacterial organisms (Fig. 3). At low concentrations of IgG a correlation was observed between bound and

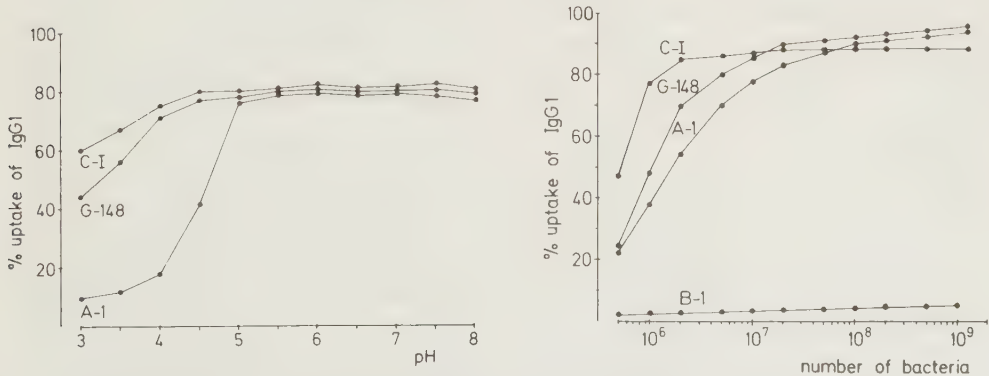


Fig. 2(A) Binding of human IgG1 at various pH. Isotope-labelled myeloma protein (0.3 μ g) was tested for binding to 2×10^8 bacteria suspended in 500 μ l 0.1 M citrate (pH 2.0–6.0) or 0.1 M phosphate buffer (pH 6.5–8.0). (B) Binding of IgG1 as a function of number of bacteria. The binding capacity of increasing amounts of test bacteria was determined with 0.3 μ g labelled immunoglobulin in 500 μ l PBSA-TWEEN buffer. The total number of bacteria was kept constant (2×10^9 organisms) by addition of IgG(Fc) negative

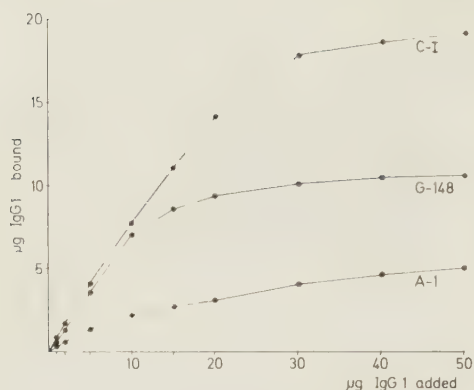


Fig. 3. Non-immune binding of human IgG1 to bacteria at different IgG concentrations. Increasing amounts of IgG1 myeloma protein were tested for binding to 5×10^8 bacteria in a final volume of 500 μ l PBSA-TWEEN buffer.

added quantities. At higher concentrations a plateau was noted. The three selected strains differed in maximal binding capacity. At a concentration of 50 μ g/500 μ l the group A and group G strains (A-1 and G-148) were capable of binding 5 and 10.5 μ g, of IgG respectively. The *S. aureus* strain Cowan I (C-I) showed an uptake of 19 μ g IgG. Similar differences were found when 25 other isolates were tested with 6 μ g labelled IgG1. Ten group A streptococci studied showed a mean uptake of 1.5 μ g IgG1 with a range of 0.9–2.1 μ g. The corresponding quantities bound by 10 group G streptococci and 5 *Staphylococcus aureus* strains were 3.6 μ g

(range 2.8–4.0) and 9.8 μ g (range 7.8–12.1), respectively.

Kinetic studies

Association kinetics. Early association events could not be resolved merely by separating the components by centrifugation. The binding step was therefore terminated by addition of unlabelled polyclonal IgG in vast excess. Displacement of bound labelled IgG1 myeloma protein was minimized by rapid cooling of the mixture before centrifugation. Experiments carried out by this procedure revealed a time-dependent process with rapid association of the components (Fig. 4 A). After 10, 30, 60 and 90 sec, uptake levels of 43, 66, 71 and 74% were noted with a human group G streptococcus (G-148). The corresponding binding levels observed with a group A streptococcus (A-1) were 40, 54, 63 and 69%. *S. aureus* (C-1) demonstrated the fastest uptake with binding levels of 74 and 81% after 10 and 30 sec.

Dissociation studies. Radiolabelled IgG1 was first reacted with bacteria in a minimal volume and then diluted 70 times with buffer. Bound immunoglobulin will dissociate until a new equilibrium is reached. In the present study the dissociation process was followed for 3 hr by determining the amount still bound to bacteria at selected time intervals. When bound immunoglobulin was plotted against time an initial phase of rapid dissociation and a second phase with

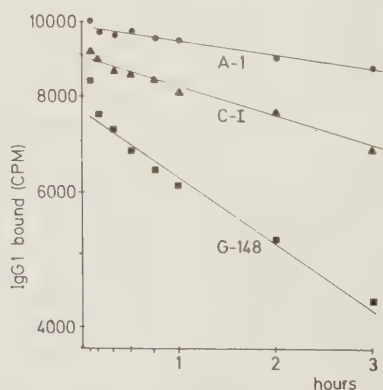
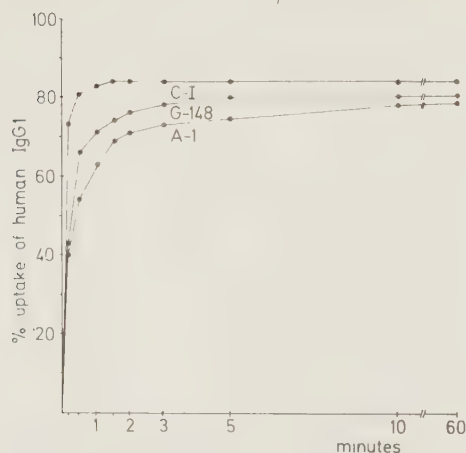


Fig. 4 (A) Rate of binding of 0.3 μ g isotope labelled IgG1 myeloma protein to 2×10^8 bacteria. The uptake was stopped at time intervals ranging from 10 sec to 60 min by addition of 1 mg unlabelled human IgG followed by centrifugation. (B) Semilogarithmic plot of the dissociation of IgG1 bound to bacteria as a function of time. Isotope-labelled IgG1 was reacted with 2×10^8 bacteria in a final volume of 50 μ l PBSA-TWEEN buffer. After 1 hr at room temperature samples were diluted 70 times with PBSA-TWEEN buffer and the amounts still bound to bacteria determined. At time 0, 0.28 μ g, 0.25 μ g and 0.22 μ g IgG1 were

Table 3. Binding of radiolabelled human Fab and Fc fragments to selected bacterial strains carrying defined types of IgG receptors^a

Immunoglobulin preparation			A-1 FcR II	G-148 FcR III	BG-6 FcR IV	Cowan I FcR I	B-1 Fc-negative
IgG polyclonal		Fab	9	9	2	5	2
		Fc	81	85	20	76	2
IgG1 And		Fab	2	61	3	2	2
		Fc	78	81	17	75	4
IgG1 Per		Fab	11	21	2	11	2
		Fc	72	75	15	70	3
IgG2 Car		Fab	3	4	2	2	2
		Fc	69	75	5	72	4

^a Binding levels are expressed as the per cent uptake of 0.2–0.4 μ g of radiolabelled protein to 2×10^8 bacteria.

slower dissociation was seen (Fig. 4 B). Regression analysis of the data comprising the second phase showed that semilogarithmic straight lines could be constructed ($r=0.97$). This implies that the dissociation process follows first order kinetics. Thus the three tested strains showed different rates of dissociation. The rate constants for strains A-1, Cowan I and G-148 were found to be 1.3×10^{-5} , 2.3×10^{-5} and 4.8×10^{-5} sec, respectively.

Binding of Fab and Fc fragments

Human polyclonal IgG and three IgG myeloma proteins were digested with papain (Table 3). Fab and Fc fragments were isolated, radiolabelled and tested for binding to human group A (A-1), group G (G-148), a bovine group G streptococcal strains (BG-6) and *S. aureus* (Cowan I) (Table 3). Fc fragments prepared from polyclonal and monoclonal IgG were similar in their reactivity with high levels of binding to A-1 (69–81%), G-148 (75–85%) and Cowan I (70–76%). A 15–20% uptake was noted with BG-6. Polyclonal Fab fragments demonstrated a weak reactivity with 9% uptake to A-1 and G-148 and 5% binding to Cowan I. The non-specific background binding assessed with a group B streptococcal strain was 2%. Monoclonal Fab fragments derived from the myeloma proteins And and Per showed a definite reactivity with group G streptococci as revealed by 61 and 21% binding, respectively, to G-148 (Table 3). Fab fragments from myeloma protein Car were completely non-reactive. The specific binding of Fab fragments from And and Per could be inhibited by polyclonal IgG but not by purified human fibrinogen or serum albumin, other serum proteins capable of interacting with streptococcal strains (Kronvall *et al.*, 1979; Måhre & Kronvall, 1980a).

Inhibition studies

Normal human serum, polyclonal human IgG, Fab and Fc fragments produced by papain digestion of human polyclonal IgG were tested for their capacity to inhibit the uptake of radiolabelled myeloma proteins to the streptococcal strains A-1 and G-148. Addition of increasing amounts of polyclonal IgG resulted in a dose-dependent inhibition of the binding of all monoclonal proteins studied. Complete inhibition of the specific uptake was noted with 200–500 μ g of inhibiting immunoglobulin. Dilutions of human serum were found to be inhibitory at concentrations corresponding to the inhibition obtained with purified IgG. Thus, other serum proteins did not seem to affect the IgG binding. Employment of isolated IgG Fc fragments resulted in a dose-related inhibition similar to that observed with intact IgG molecules. No inhibition was found when Fab fragments derived from polyclonal IgG were used.

The inhibiting capacities of four isolated myeloma proteins were compared in a test system under completely identical circumstances. The selected myeloma proteins Hed, Blo, Sve and Far represent various IgG subclasses (Table 1). Various amounts of purified monoclonal protein were mixed with a labelled test protein and the uptake to two streptococcal strains determined (Fig. 5). All four preparations were inhibitory but differed in potency. Studies performed with the strain A-1 showed that Hed (IgG1) was strongly inhibitory in contrast to the more moderate reduction in uptake noted with Blo (IgG3) and Far (IgG4) (Fig. 5A). When G-148 bacteria were used a different pattern was observed. Blo and Hed were potent inhibitors, in contrast to Far and Sve which were less reactive (Fig. 5B).

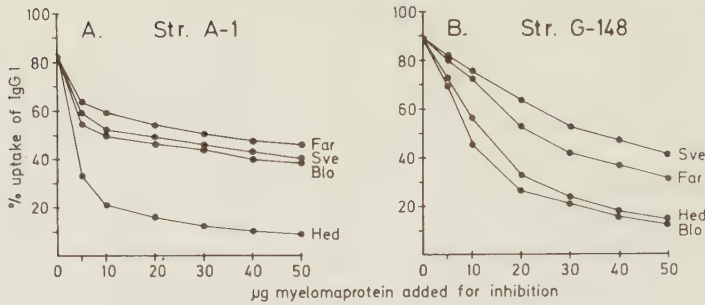


Fig. 5. Capacity of unlabelled monoclonal proteins to compete with radiolabelled standard IgG for binding to streptococcal strains A-1 (Fig. 4 A) and G-148 (Fig. 4B). The monoclonal components Hed, Blo, Sve and Far represent IgG subclasses 1, 2, 3 and 4, respectively. The uptake of radiolabelled IgG is plotted against amount of monoclonal protein added for inhibition.

Scatchard analysis of binding data

The bacterial strains A-1, G-148 and Cowan I were tested for binding of varying amounts of IgG1 (monoclonal protein Hed). Bound (b) and free (f) fractions were calculated and the data plotted as described by Scatchard (1949) using the equation $b/f = nK - bK$. Strains A-1 and G-148 demonstrated non-linear plots with a linear section at b/f ratios higher than 0.6 (Fig. 6A). The association constants derived from the linear section of the plots were 9.4×10^7 and 7.8×10^7 1/mole for A-1 and G-148, respectively. A straight regression line could be drawn for Cowan I (Fig. 6B). The association constant measured with Cowan I was 5.1×10^7 1/mole.

DISCUSSION

Existence of specific binding sites for human IgG on the streptococcal cell surface is well established (Kronvall, 1973; Myhre & Kronvall,

1977; Myhre *et al.*, 1979; Myhre & Kronvall, 1979; Freimer *et al.*, 1979). The present investigations show that human group A, C and G streptococci can bind all four human IgG subclasses. Our results confirm earlier observations (Kronvall, 1973; Christensen & Oxelius, 1974) and are consistent with the high levels of uptake of polyclonal IgG noted in previous studies (Myhre & Kronvall, 1977). This broad reactivity with binding of all IgG subclasses differs from the restricted specificity of related non-human streptococcal species. Equine group C streptococci (*Streptococcus zooepidemicus*) exhibit a protein A-like reactivity with binding of human IgG1, IgG2 and IgG4, and β -hemolytic bovine group G streptococci interact mainly with human IgG1 and IgG2 (Table 2). Group A streptococci (IgG-Fc receptor type II) do not differ from human group C and G streptococci (IgG-Fc receptor type III) in their specificity for human IgG subclasses.

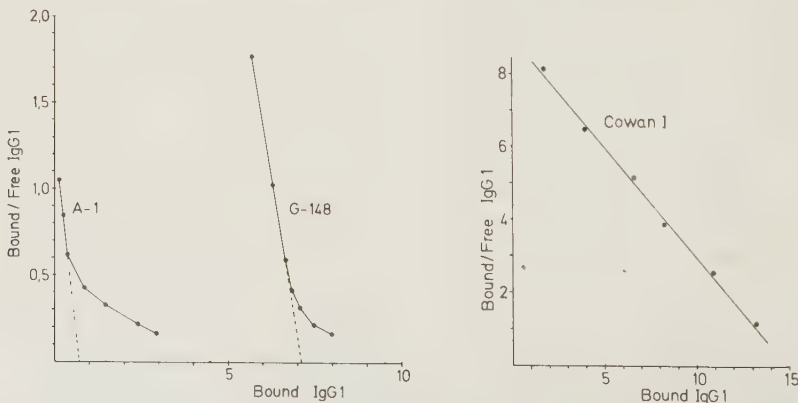


Fig. 6. Scatchard analysis of binding experiments performed with bacterial strains A-1, G-148 and Cowan I. Increasing amounts of (2–50 μ g) radiolabelled IgG1 were mixed with 5×10^8 bacteria in 700 μ l PBSA-TWEEN buffer. Bound (b) and free (f) fractions were determined and the ratio b/f plotted against the

However, these two receptor types demonstrate different binding patterns for bovine and murine IgG (Myhre & Kronvall, 1979; Myhre & Kronvall, 1980 b).

The molecular basis for the binding of IgG to staphylococcal protein A has recently been studied by crystallography. These studies showed that the Fc-reactive fragment B of protein A provides two areas of contact with IgG-Fc, one extensive hydrophobic area and another smaller area involving a few polar residues (Deisenhofer *et al.*, 1978). Histidine 435, an external polar residue, seems to be essential for the protein A reactivity (Deisenhofer *et al.*, 1978). In the protein A negative IgG3 subclass this residue is exchanged for arginine (Michaelsen *et al.*, 1977; Wolfenstein-Todal *et al.*, 1976). The present investigations showed that the streptococcal IgG(Fc) receptors type II and III exhibit high avidity for all four human IgG subclasses. It seems reasonable to conclude that the interaction with the streptococcal receptors has a molecular basis different from that described for protein A. The unrestricted binding of IgG subclasses indicates that a basic structure common to all four isotypes of γ chains is involved in the streptococcal reactivity.

In a series of experiments, unlabelled monoclonal proteins were tested for their capacity to compete with a radiolabelled standard IgG preparation for binding to type II and III binding sites (Fig. 5). The fact that these myeloma proteins representing the four IgG subclasses were all inhibitory indicates that these subclasses are bound to the same streptococcal surface structure. However, individual myeloma proteins differed in their inhibiting capacity. Differences were also noticed between the two bacterial strains studied (Fig. 5). For example, the IgG3 myeloma protein Blo [G3 m(5)], which was highly inhibitory in the G-148 test system, was only moderately inhibitory in the A-1 system. The homogenous nature of the myeloma proteins suggests that this type of heterogeneity reflects the existence of slight variations among IgG receptors in different strains. The limited number of monoclonal proteins included in the present studies does not allow any conclusion to be made as to whether isotypic or allotypic determinants are responsible for this variability. Similar observations have been made by other investigators (Kronvall, 1973; Schalén *et al.*, 1978). For example, IgG binding structures extracted from a group A streptococcus were found to exhibit some degree of allotype

specificity with strong binding of G3m (5) myeloma proteins, in contrast to a much weaker interaction with G3m (21) monoclonal proteins (Schalén *et al.*, 1978). The differences noted in our studies could have a similar basis.

Streptococcal IgG receptors on the bacterial cell surface are susceptible to digestion with proteolytic enzymes (Myhre & Kronvall, 1980 c). The immunoglobulin receptors show a localization separate from the binding sites for other serum proteins capable of interaction with streptococci. It is not known whether the IgG receptors are evenly distributed over the bacterial cell surface or localized to areas of high receptor density. Scatchard analysis of binding experiments performed with myeloma proteins labelled by an extremely mild enzymatic procedure resulted in non-linear plots (Fig. 6A). The homogenous nature of the ligand suggests functional heterogeneity of the binding sites. The curvo-linear plots observed imply that a certain amount of IgG binds with high avidity and that further uptake involves binding sites of lower avidity. The excessive release of bound immunoglobulin during the early phase of the dissociation could represent such less avidly bound material.

Experiments performed with isolated Fab and Fc fragments demonstrated that the streptococcal reactivity resides in the Fc portion of the immunoglobulin molecule. Fc fragments were as effective as intact IgG molecules in their inhibiting capacity, indicating that the Fab portion is not appreciably involved in the binding of IgG. However, Fab fragments from two IgG myeloma proteins showed definite reactivity with streptococci carrying Fc receptors of type III. Although a regular immune binding cannot be ruled out, this activity could represent a new type of interaction normally concealed by the dominating Fc reactivity. Other investigators have recently reported a similar interaction between staphylococcal protein A and porcine IgG Fab fragments (Milon *et al.*, 1978; Endresen, 1979).

The property of Fc-reactive bacteria to interact with immunoglobulins irrespective of their antigen specificity has been exploited in immunological work (Goding, 1978). Protein A-containing staphylococci have been used as solid-phase adsorbent for the isolation and identification of mammalian membrane antigens in combination with specific antisera (Kessler, 1975; Kessler, 1976; Cullen & Schwartz, 1976), for detection of immune complexes (Tucker *et*

al., 1978; McDaugal *et al.*, 1979) and for preferential IgG adsorption in serological methods for demonstration of IgM antibodies (Ankerst *et al.*, 1974; Skaug & Tjoetta, 1974; Jonsson & Nordenfelt, 1979). The capacity of streptococci to bind all four human IgG subclasses, the high avidity of the binding and the stability of heat treated bacteria are features which suggest that selected group C and G streptococci can become even more valuable tools for the immunologist.

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VI

Specific binding of bovine, ovine, caprine and equine IgG subclasses to defined types of immunoglobulin receptors in Gram-positive cocci

Erling B. Myhre and Göran Kronvall

Department of Medical Microbiology, University of Lund,
Lund, Sweden

Short title: Immunoglobulin binding to bacteria

ABSTRACT

One hundred and twenty bacterial strains were tested for non-immune binding of radiolabelled bovine, ovine, caprine and equine immunoglobulins. Bacteria possessing previously defined IgG receptors interacted in a well defined manner with purified IgG subclass immunoglobulins. Human group C and G streptococci carrying IgG receptors type III were capable of binding all IgG subclasses in the four mammalian species studied. Protein A-containing staphylococci demonstrated a restricted specificity with binding of bovine IgG1, ovine IgG1, caprine IgG1 and IgG2 as well as equine IgG(ab). Group A streptococci which can bind human IgG did not show specific reactivity. A new type of binding unrelated to the regular Fc-mediated binding was observed with equine IgG(T).

The differences in specificity for IgG subclasses suggest that structures with binding capacity to streptococcal type III Fc receptors are different from staphylococcal protein A reactive sites. Inhibition experiments performed with purified immunoglobulins showed that individual IgG subclasses differed greatly in their inhibiting capacity reflecting differences in avidity.

The high avidity and the broad, unrestricted immunoglobulin G reactivity of streptococcal IgG receptor type III indicate that human group C and G streptococci may provide a valuable tool for solid phase absorption of immunoglobulins from several mammalian species.

Key words: Group A, C and G streptococci, IgG(Fc) receptors, mammalian IgG subclasses, non-immune reactivity.

Réactivité spécifique des sous-classes IgG bovine, ovine, caprine et chevaline à différents types de récepteurs d'immunoglobine dans les coques à gramme positif.

Cent vingt souches de bactéries ont été testées concernant la réactivité non immunisée d'immunoglobines iodées bovines, ovines, caprines et chevalines. Les bactéries possédant des récepteurs IgG définis antérieurement, sont entrées en interaction d'une manière très définie avec la sous-classe IgG d'immunoglobines purifiées. Les streptocoques du groupe humain C et G portant des récepteurs IgG de type III ont été capables de réaction avec toutes les sous-classes IgG des quatre espèces mammaliennes étudiées. Les staphylocoques contenant la protéine A ont montré une spécificité limitée de réaction avec l'IgG bovine, l'IgG ovine, l'IgG1 et l'IgG2 caprines, aussi bien qu'avec l'IgG(ab) chevaline. Les streptocoques du groupe A qui peuvent réagir avec l'IgG humaine n'ont pas montré de réactivité spécifique. Un nouveau type de réactivité, sans liaison avec la réaction régulière opérée par la molécule FC a pu être observée avec l'IgG(T) chevaline.

Les différences de spécificité concernant les sous-classes IgG montrent que les structures ayant une capacité de réactivité aux récepteurs streptocociques de type III FC sont différentes des zones de réactivité de la protéine A staphylococique. Les expériences d'inhibition réalisées avec des immunoglobines purifiées ont montré que les sous-classes IgG individuelles diffèrent de façon marquée dans leur capacité d'inhibition, montrant des différences d'avidité.

La grande avidité et la haute réactivité, sans restriction de l'immunoglobine G du récepteur streptococique de type III montre que les streptocoques du groupe humain C et G peuvent fournir un instrument valable pour une importante phase d'absorption des immunoglobines de différentes espèces mammaliennes.

Mots-clefs: Streptocoques du group A, C et G; récepteurs IgG(Fc); sous-classes mammaliennes IgG; réactivité non immunisée.

INTRODUCTION

In 1958 Jensen described a staphylococcal cell wall antigen, later designated protein A, which precipitated with normal human serum (1). Forsgren and Sjöquist found that this reactivity was mediated by sites on the Fc-portion of the immunoglobulin G molecule (2). An analogous reactivity was later detected in β -hemolytic streptococci of Lancefield groups A, C and G (3). Our initial studies have shown that non-immune Fc reactivity is mediated by three major types of IgG(Fc) receptors present on the bacterial cell surface: Type I represented by staphylococcal protein A, type II restricted to group A streptococci and type III carried by human β -hemolytic group C and G streptococci (4).

Two additional types of IgG receptors have recently been described: one present on β -hemolytic bovine group G streptococci (5) and another found on Streptococcus zooepidemicus (6).

Bacterial IgG receptors interact not only with human IgG but also with immunoglobulins from many mammalian species (7, 8). Studies of protein A reactivity have revealed that the reactivity within a species may be restricted to certain IgG subclasses (9-12). Similar studies have not yet been performed with immunoglobulin binding streptococci. The aim of the present investigation was therefore to explore systematically the specificity of previously defined IgG receptors in Gram-positive cocci. Cattle, sheep, goat and horse, species of veterinary importance, were selected for these experiments. Antigenically and functionally distinct IgG subclasses have been described in these species (13-17).

Our studies show that the streptococcal IgG receptor type III has a very broad reactivity, with positive binding of all IgG subclasses tested. This broad reactivity suggests that human group C and G streptococci may become useful tools in immunological work for solid phase absorption of immunoglobulins from several mammalian species.

MATERIAL AND METHODS

BACTERIAL STRAINS

A total of 120 bacterial strains representing 10 different species were studied: S. aureus, group A, B, C (group C species: Strep. equisimilis, dysgalactiae, zooeptidemicus and equii) and group G streptococci (human and bovine β -hemolytic strains and bovine α -hemolytic strains). Bacterial isolates were maintained by repeated subcultures on horse blood agar plates. For binding studies staphylococcal and streptococcal strains were grown in Tryptone and Todd-Hewitt broth, respectively.

IMMUNOGLOBULIN PREPARATIONS

IgG subclasses from four different mammalian species were included in the present study.

Bovine IgG: Purified bovine IgG1 and IgG2 were purchased from Miles Laboratories, Inc., Elkhart, Indiana, USA.

Ovine and caprine IgG: Serum samples from unimmunized animals were subjected to ion exchange chromatography. Both ovine and caprine IgG2 do not bind to DEAE-cellulose equilibrated with 0.01 M phosphate buffer pH 8.0 (14-15). Immuno-electrophoretic analysis showed that the fall-through peak from a DEAE-Sephacel column contained only IgG. This material did not precipitate

with an antiserum with specificity for bovine IgG1. It was therefore designated ovine and caprine IgG2, respectively. Fractions eluted from the ion exchange matrix with a NaCl gradient were tested for precipitation with rabbit anti-IgG antisera. Strongly precipitating fractions were pooled and concentrated by ultrafiltration (Amicon, Diaflow ultrafilter PM-30). Pooled material precipitated with an anti-bovine IgG1 specific antiserum but was contaminated with serum globulins other than IgG. Contaminating proteins were removed by gel filtration on a Sephadex G-200 column (2.6 x 80 cm) operated at 4°C with a continuous flow of 10 ml phosphate buffer (PBSA, 0.12 M sodium phosphate, 0.03 M NaCl, 0.02 per cent sodium azide, pH 7.2) per hour. Fractions containing monomeric immunoglobulin were pooled, concentrated and referred to as IgG1. Purified ovine and caprine IgG subclass preparations demonstrated characteristic electrophoretic mobilities as reported by Feinstein and Hobart (16).

Equine IgG: Equine immunoglobulins were isolated from horse serum using currently recommended procedures including ion exchange chromatography, preparative block electrophoresis and gel filtration (18). Pooled, normal horse serum was precipitated with 40 per cent saturated ammonium sulphate, resuspended in PBSA and then dialyzed extensively against 0.01 M sodium phosphate buffer, pH 8.0. Seven ml of dialyzed protein solution (30 mg per ml) were fractionated on DEAE-Sephacel as described below. Fractions constituting the ascending limb of the initial protein peak were pooled and concentrated. Under the experimental conditions this pool contained pure IgG(ab). Chromatography fractions eluted with a sodium chloride gradient were tested in an agarose gel for precipitation against anti horse IgG and anti horse IgG(T) specific antisera. Fractions positive for IgG but free of IgG(T) were pooled, concentrated and subjected to block electrophoresis (0.6 per cent agarose gel, 0.0075 M barbital buffer pH 7.4) (19) to remove contaminating IgG(ab). IgG(T) rich chromatography fractions were also pooled, concentrated and separated by agarose block

electrophoresis. Final purification was accomplished by gel filtration on a Sephadex G-200 column. The IgG(c) preparation contained small amounts of other serum globulins as revealed by immunoelectrophoresis. When this preparation was radiolabelled and precipitated with monospecific anti horse IgG antiserum, more than 75 per cent of the isotope was detected in the precipitate. The identity of the various equine IgG subclasses was confirmed by studies kindly performed by Dr. W.E. Bernadina, employing specific antisera.

IgG concentrations were measured by a commercially available dye-binding assay using bovine IgG as a protein standard (bio-Rad protein assay, Bio-Rad laboratories, Richmond, California, USA).

ANTISERA

Antisera with specificity for bovine, ovine and caprine IgG as well as antisera to equine IgG(T) and equine serum proteins were purchased from Miles Laboratories. Antisera to bovine, ovine and caprine serum proteins were obtained from DAKO-immunoglobulins Ltd, Copenhagen, Denmark. Antisera to bovine IgG1 and IgG2 (Miles Laboratories) crossreacted with ovine and caprine IgG subclasses.

BINDING ASSAY

Overnight broth cultures were washed and suspended as described previously (4). Quantitative binding assays were performed in triplicates as described in earlier studies by mixing 0.5 µg labelled immunoglobulin with 2×10^8 bacteria in a final volume of 225 µl PBSA-Tween buffer (4). The IgG uptake was expressed as the percentage of added radioactivity bound to bacteria deposited by centrifugation. The binding to polystyren test tubes in the absence of bacteria was less than 1 per cent. For inhibition experiments 0.5 µg radiolabelled

immunoglobulin was mixed with increasing amounts of purified IgG preparations. The mixtures were then incubated with bacteria in a final volume of 500 μ l and the uptake of radioactivity measured.

RADIOLABELLING

Purified immunoglobulin preparations were labelled with I^{125} (code no IMS 30, The Radiochemical Centre, Amersham, U.K.) using lactoperoxidase coupled covalently to Sepharose (20). Incorporation of isotope into protein was estimated by paper chromatography (21). Free, unreacted isotope was removed by extensive dialysis against PBSA containing 0.05 per cent Tween 20 (PBSA-Tween buffer). Labelled preparations showed specific activities of 10-20 MBq per mg protein. Labelled immunoglobulin preparations were mixed with normal serum and analyzed by immunoelectrophoresis using species specific antisera. Autoradiographic analysis of the slides showed that the radiolabel was associated only with the IgG precipitate.

AFFINITY CHROMATOGRAPHY

Purified IgG subclass immunoglobulins were also tested for binding to protein A covalently bound to Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden). Samples containing between 2.1 and 5.9 μ g IgG were applied to a 0.8 x 2.4 cm column and eluted at 4°C at a speed of 1 ml per hour with PBSA containing 0.1 per cent human serum albumin (HSA). After eight column volumes of start buffer adsorbed immunoglobulins were eluted with 0.1 M glycine -HCl buffer, pH 2.8, containing 0.1 per cent HSA. The radioactivity of 0.5 ml collected fractions was measured in a gamma counter. Control experiments were performed under similar conditions on Sepharose CL-4B.

RESULTS

QUANTITATIVE BINDING OF IMMUNOGLOBULINS

One hundred and twenty bacterial strains belonging to ten different species of Gram-positive cocci were tested for binding of bovine, ovine, caprine and equine IgG subclasses. These studies were performed with a sensitive technique employing purified radiolabelled immunoglobulin preparations. Several isolates of each species were included because earlier studies have demonstrated some variability between individual strains.

Staphylococcus aureus: All fifteen protein A carrying strains (IgG receptor type I) were homogenous in their reactivity. High binding levels were observed with bovine IgG2 (59 %) and ovine IgG2 (60 %) (Table 1). Uptake of bovine IgG1 and ovine IgG1 was comparable to that seen with group B streptococcal strains which were included as negative control strains. Both caprine IgG subclasses were reactive with 30 and 60 % binding of IgG1 and IgG2, respectively (Table 1). Equine IgG(ab) exhibited a low degree of reactivity with an uptake level of 19 %. Equine IgG(c) and IgG(T) did not bind to staphylococci.

Group A streptococci: None of the examined group A strains showed any significant reactivity (Table 1). Binding of bovine, ovine and caprine IgG did not exceed that bound by negative group B streptococci. Equine IgG(ab) and IgG(c) were also non-reactive. Thirteen out of 16 strains interacted with equine IgG(T) with uptakes ranging from 20 to 51 %.

Streptococcus equisimilis: Fifteen of 16 strains tested exhibited broad reactivity with positive binding of all studied IgG subclasses (Table 1). The average uptake of bovine, ovine and caprine IgG1 (77, 79 and 81 %, respectively) was lower than that noted with the corresponding IgG2 subclasses

(89, 85 and 90 %). Equine IgG(ab) was strongly reactive as reflected by an uptake level of 81 %.

In contrast equine IgG(c) and IgG(T) showed only 23 and 24 per cent binding. Individual strains varied greatly in their reactivity. However, strains which interacted strongly with one immunoglobulin preparation were also potent binders of the other subclass proteins. Thus, none of the strains studied demonstrated selective binding of any subclass.

Streptococcus dysgalactiae: Seven of 10 strains were reactive with a binding pattern similar to that noted with the closely related species Streptococcus equisimilis (Table 1). Positive binding was noted with all preparations. High levels of uptake were recorded with bovine, ovine and caprine IgG2. The corresponding IgG1 subclasses were slightly less reactive with lower uptake levels. Equine IgG(ab) demonstrated high reactivity in contrast to a low degree observed with the subclasses IgG(c) and IgG(T).

Streptococcus zooepidemicus: Eight of 10 strains were capable of binding small but significant amounts of all studied subclass preparations (Table 1). Bovine IgG2 and equine IgG(ab) were more reactive than the other immunoglobulin preparations.

Streptococcus equii: All strains interacted weakly with bovine IgG2 as well as the three equine IgG preparations (Table 1). Bovine IgG1 and the two ovine and caprine IgG subclasses were completely negative.

Human group G Streptococci: Thirteen out of 15 strains presented a picture identical to the binding pattern of Streptococcus equisimilis strains (human group C streptococci) (Table 1). A broad reactivity with binding of all bovine,

ovine, caprine and equine IgG subclasses was found. The binding of IgG1 was always lower than the IgG2 uptake. Whereas equine IgG(ab) showed a 73 % binding only 21 % IgG(c) and IgG(T) were bound under the same test circumstances.

Bovine group G streptococci: Two distinct non-human group G streptococcal species were studied. An interaction between β -hemolytic strains and all three equine IgG subclasses was noted (Table 1). Higher binding levels were, however, obtained with IgG(T) than with IgG(ab) and IgG(c). Immunoglobulins from other species were non-reactive.

Although individual strains differed in their reactivity (intraspecies differences) the bacterial species included demonstrated characteristic binding patterns.

In the initial experiments equine IgG(T) interacted with most bacterial species including group A streptococci which did not bind other subclass preparations. This atypical reactivity was further explored by comparison of the binding of the three equine IgG subclasses to human group C and G streptococci. The uptake levels of IgG(c) and IgG(T) were plotted against the IgG(ab) binding. A definite relationship was observed between the IgG(c) and the IgG(ab) reactivity. A similar relationship could not be demonstrated for IgG(T) and IgG(ab).

INHIBITION EXPERIMENTS

Differences in immunoglobulin reactivity noted in the binding experiments were also explored in inhibition experiments. Addition of polyclonal human IgG resulted in a dose-related inhibition of the uptake of radiolabelled IgG(ab) and IgG(c) to a human group G streptococcus strain (G-148). In contrast, human IgG at concentrations up to 500 μ g per 500 μ l had no effect on the binding of IgG(T) (Fig 1)

Studies were also designed to explore the relative receptor affinity of ruminant IgG subclasses. A Staphylococcus aureus strain (Cowan I) and a β -hemolytic human group G streptococcus (G-148) were selected for these experiments. The two strains carry distinct types of IgG(Fc) receptors. Increasing amounts of bovine, ovine and caprine IgG subclasses were mixed with a minute quantity of radiolabelled human IgG and the uptake of isotope to bacteria determined. Studies performed with protein A carrying Cowan I strain revealed that addition of bovine, ovine and caprine IgG resulted in a dose-dependent inhibition (Fig 2A). However, the three immunoglobulin preparations differed in their inhibiting capacity. Ovine IgG2 was more potent than bovine and caprine IgG2, but they were all inferior to human polyclonal IgG included as a reference immunoglobulin. Expressed on a quantitative basis 14, 38 and 52 μ g of ovine, caprine and bovine IgG2, respectively, were required for a 50 % inhibition. The corresponding quantity human IgG was 3 μ g. Caprine IgG1 was weakly inhibitory when 100 to 150 μ g amounts were used. Bovine and ovine IgG1 exhibited no inhibitory capacity. The group G streptococci strain G-148 binds less IgG than the strain Cowan I. The following experiments were therefore performed with 10^8 streptococcal organisms and immunoglobulin quantities ranging from 0.8 to 800 μ g. A definite but varying inhibition was obtained with all six IgG subclass preparations (Fig 2B). Bovine IgG demonstrated the highest degree of inhibiting capacity followed by ovine IgG2, caprine IgG2, caprine IgG1, bovine IgG1 and ovine IgG1. Thus the IgG2 subclasses were generally more potent than the corresponding IgG1 preparations. The amount of IgG2 immunoglobulins (5-7 μ g) which resulted in a 50 per cent inhibition was comparable to the quantity human reference IgG required. In contrast 14-80 μ g of IgG1 immunoglobulins had to be added to achieve the same degree of inhibition.

AFFINITY CHROMATOGRAPHY

Binding assays performed with S. aureus organisms were extended to affinity chromatography studies on protein A-Sepharose. These experiments were carried out with small quantities of purified radiolabelled IgG subclass immunoglobulins (Table 2). More than 90 % of bovine IgG2, ovine IgG2 and caprine IgG2 were bound and eluted with 0.1 M glycine pH 2.8. Bovine and ovine IgG1 which were negative in the direct binding assay, showed a low degree of reactivity. Both preparations presented an elution pattern with a very broad fall-through peak and a continuous release of immunoglobulin during the elution with eight column volumes of start buffer. Forty per cent of bovine IgG1 and 30 per cent of ovine IgG1 was finally eluted with acid glycine. Both caprine IgG subclasses exhibited reactivity with specific binding of 73 and 91 per cent of caprine IgG1 and IgG2, respectively. The protein A reactivity of equine IgG(ab) noted in the binding experiments was reflected in specific uptake of 54 % of the test amount to protein A-Sepharose (Table 2). A small but continuous release of IgG(ab) was seen during elution with start buffer. Equine IgG(T) did not interact with protein A-Sepharose.

DISCUSSION

Non-immune binding of immunoglobulin to Gram-positive cocci is mediated by specific binding sites, IgG receptors, present on the bacterial cell surface (4). The present investigation confirms the existence of distinct types of IgG receptors previously defined by differences noted in inhibition studies employing a panel of animal sera (4). Our studies show that these IgG receptor types interact in a well defined manner with bovine, ovine, caprine and equine subclasses. Two distinct IgG subclasses, IgG1 and IgG2, are found in the ruminant species cow, sheep and goat (13-16). Horse IgG is composed of three subclasses termed IgG(ab), IgG(c) and IgG(T) (17-18).

The IgG receptor type III found in human group C and G streptococci interacts with all IgG subclasses of these four species. Such a broad reactivity with binding of all four human IgG subclasses was noted in earlier studies (22). In contrast, group A streptococci (IgG receptor type II) can also combine with all four human IgG subclasses but are incapable of binding non-human immunoglobulins. Similarly, bovine group G streptococci (IgG receptor type IV) can bind small but definite amounts of human IgG but do not exhibit any affinity for mammalian IgG. The restricted specificity of staphylococcal protein A reported by other investigators was confirmed (8, 12). Protein A reactivity can be detected in bovine IgG2, ovine IgG2, caprine IgG1, caprine IgG2 and equine IgG(ab). Bovine IgG1 and ovine IgG1 have previously been considered protein A negative, but our present chromatographic studies indicate that these two subclasses have a low but detectable reactivity (Table 2). Studies performed with human IgG myeloma proteins have shown that Streptococcus zooepidemicus carries protein A-like IgG receptors (6). In the present investigation this receptor type differed substantially from protein A both with respect to binding capacity and subclass specificity (Table 1) and therefore represents a distinct type of Fc receptor. Streptococcus equii, a group C streptococcal species, showed a binding pattern which did not resemble any of the other species. Thus Strep. equii may possess a reactivity different from that described in related bacterial species.

The structure on the immunoglobulin molecule involved in the non-immune binding to the bacterial cell has not been delineated, but previous studies have shown that the reactivity resides in the Fc-portion of the molecule (3, 22, 23). When the capacity of purified ruminant immunoglobulins to inhibit the uptake of human IgG to selected test strains was determined, considerable variability was noted between closely related IgG subclasses. These findings imply that the binding

site present on related IgG subclasses differs slightly, resulting in different affinities for the receptor. It is interesting to note that the IgG2 subclasses in the three ruminant species seem to be more inter-related to each other than to their corresponding IgG1 subclass. This corresponds with the observation that antisera specific for ovine IgG1 and IgG2 react only with corresponding subclasses of cattle and goat (16). The universal reactivity of human group C and G streptococci with binding with all IgG subclasses, and the restricted reactivity noted with protein A carrying staphylococci, suggest that the immunoglobulin structure binding to type III receptors is distinct from the protein A reactive site. Type II receptors of group A streptococci seem to recognize still another structure found only on human immunoglobulin molecules.

Equine IgG(T) showed an atypical reactivity with binding to all bacterial species including the otherwise negative group A streptococci (Table 1). The uptake of IgG(T) to bacteria did not correlate with the binding of equine IgG(ab) and IgG(c). Furthermore, human polyclonal IgG which inhibited the binding of the IgG(ab) and IgG(c), had no effect on the uptake of IgG(T). These features suggest that a reactivity different from the Fc-mediated immunoglobulin reactivity was responsible for binding to Gram-positive cocci. Equine IgG(T) has distinct functional properties including an inability to precipitate with antigen (24) and was for a long time regarded as a separate immunoglobulin class. Studies on the amino acid sequence in the C-terminal portion of the molecule have, however, revealed considerable homology with the other IgG subclasses in the horse, and the T component is now classified as an IgG subclass (18, 25).

Immunoglobulin binding bacteria have many applications in immunology. Intact staphylococci can be used to isolate immune complexes, membrane antigens, receptors and to replace "second antibody" in radioimmunoassay (26). One obvious limitation in the use of staphylococci is the poor reactivity for ruminant immunoglobulins. The protein A negative IgG1 subclass in cattle constitutes approximately 45 per cent of the total IgG(27). Human group C and G streptococci with their capacity to bind both subclasses do not have this drawback. Such bacteria can bind substantial amounts of IgG very rapidly (22). These features suggest that selected strains of human streptococci may become valuable tools in the hands of the veterinary immunologist.

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Table 1. Binding of purified IgG subclasses to *S. aureus*, group A, C, G and β streptococci. Binding capacities are expressed as per cent uptake of 0.5 μ g isotope-labelled immunoglobulin to 2×10^8 bacterial organisms *.

Immuno- globulin	<u>Staphylo- coccus aureus</u>	<u>Group A strept.</u>	<u>Strept. equisi- milis</u>	<u>Strept. dysga- lactiae</u>	<u>Strept. zooepi- demicus</u>	<u>Strept. equii</u>	Human β - hemolytic group G strept.	Bovine β -hemoly- tic group G strept.	Bovine α -hemoly- tic group G strept.
Bovine IgG1	6(5-7)	5(2-6)	77(26-88)	40(4-79)	10(8-13)	8(6-9)	64(4-88)**	4(4-5)	4(3-5)
Bovine IgG2	59(49-72)	4(2-7)	89(54-95)	75(13-95)	26(18-30)	22(15-37)	83(19-94)	4(3-5)	4(3-5)
Ovine IgG1	8(9-11)	3(2-4)	79(5-87)	28(2-87)	14(6-20)	2(2-3)	71(4-90)	2(2-3)	2(2-3)
Ovine IgG2	60(55-66)	6(3-9)	85(4-93)	56(2-93)	18(8-20)	2(2-3)	79(4-95)	3(2-5)	2(2-3)
Caprine IgG1	30(27-34)	4(2-5)	81(3-92)	42(2-86)	12(7-18)	3(2-4)	72(2-90)	2(1-4)	3(2-4)
Caprine IgG2	60(56-68)	7(3-9)	90(3-97)	54(2-91)	15(9-23)	4(2-6)	82(3-95)	3(2-4)	3(2-4)
Equine IgG(ab)	19(13-22)	9(7-12)	81(54-86)	68(15-84)	39(3-56)	32(14-40)	73(18-86)	14(12-17)	7(4-12)
Equine IgG(c)	6(3-10)	8(6-11)	23(13-29)	19(7-26)	16(15-20)	15(8-20)	21(7-29)	12(10-13)	8(6-12)
Equine IgG(T)	6(3-8)	31(5-51)	24(14-39)	32(10-52)	29(12-50)	36(33-40)	21(9-40)	29(12-40)	10(2-32)
No. of reactive strains	15/15	13/16	15/16	7/10	8/10	7/7	13/15	15/15	4/6
No. of tested strains									

* Results obtained with immunoglobulin reactive strains were pooled for each bacterial species, and are shown as the arithmetic mean and the range of binding levels.

Table 2. Binding of radiolabelled IgG subclass immunoglobulins to protein A-Sepharose

Immunoglobulin	Quantity tested μg	Per cent eluted with	
		PBSA-HSA*	glycine -HCl**
Bovine IgG1	4.1	60	40
Bovine IgG2	5.9	7	93
Ovine IgG1	3.8	70	30
Ovine IgG2	4.3	4	96
Caprine IgG1	4.8	27	73
Caprine IgG2	5.2	9	91
Equine IgG(ab)	2.7	46	54
Equine IgG(c)	2.1	91	9
Equine IgG(T)	2.3	93	7

Chromatography performed at 4°C with a column containing 2.1 ml protein A-Sepharose.

* PBSA-HSA = 0.12 M phosphate, 0.03 M NaCl, 0.02 % sodium azide, 0.1 % human serum albumin, pH 7.2.

** 0.1 M glycine-HCl, pH 2.8.

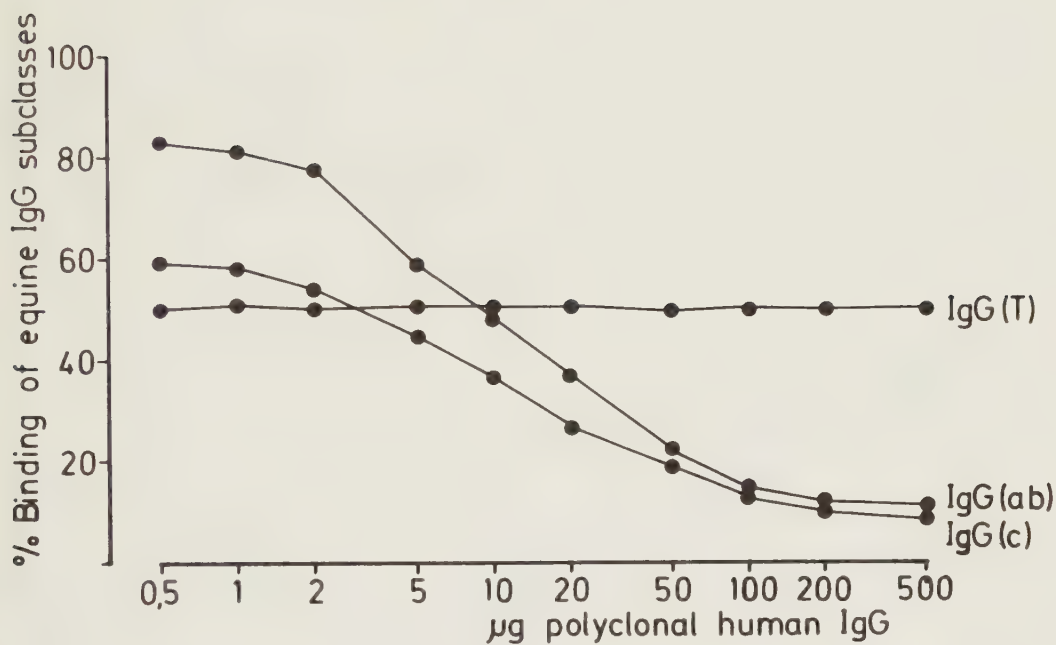


Fig 1. Capacity of polyclonal human IgG to inhibit the uptake of isotope-labelled equine IgG subclasses (ab, c and T) to the human group C streptococcal strain G-148 (2×10^8 bacterial organisms).

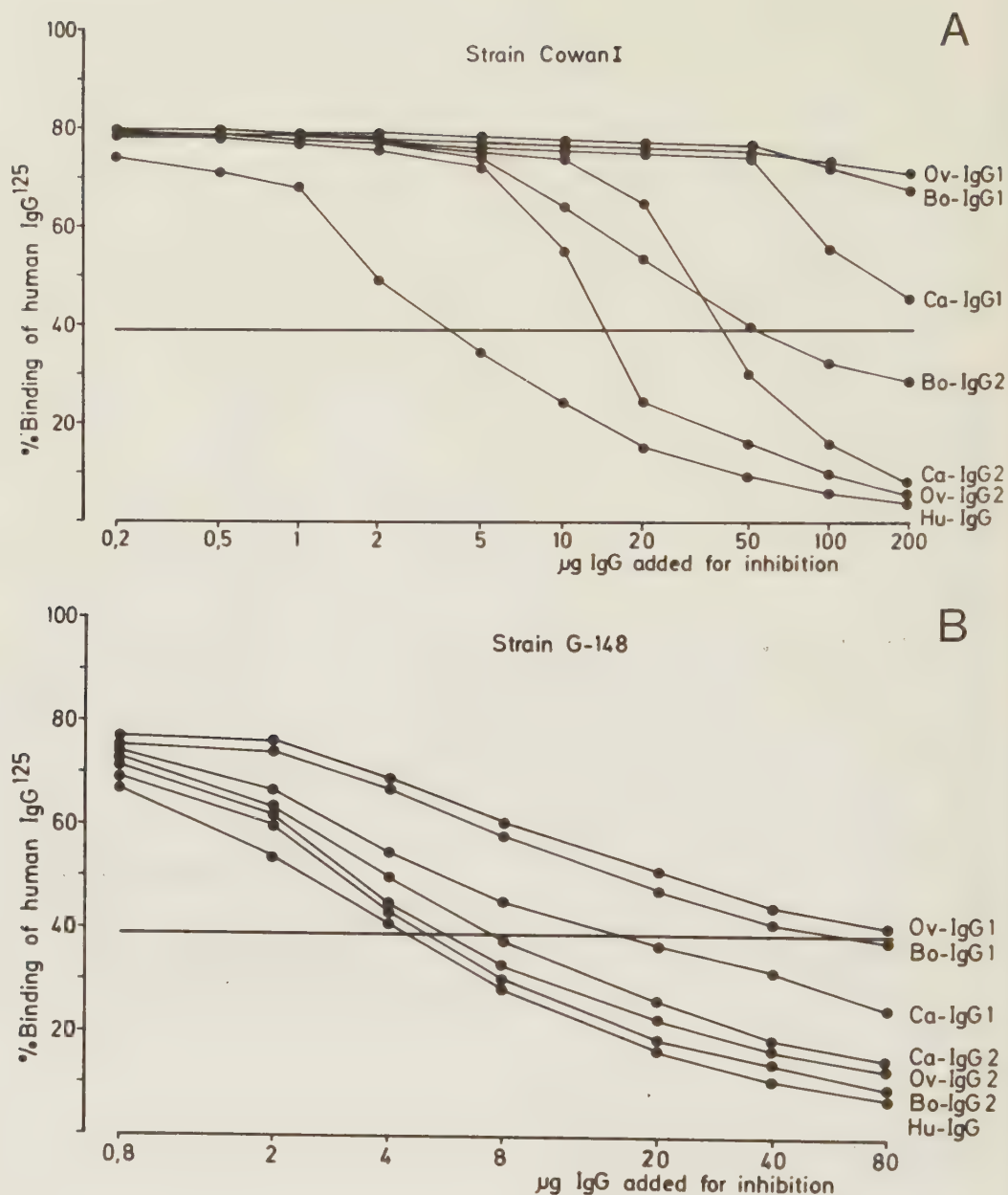


Fig 2. Capacity of purified IgG subclass immunoglobulins to compete with isotope-labelled polyclonal IgG for binding to *S. aureus* (Fig 2A) and human group G streptococci (Fig 2B). Abbreviations Bo, Ov and Ca denote purified bovine, ovine and caprine IgG subclasses. Unlabelled polyclonal human IgG (Hu) was included as a reference immunoglobulin. A horizontal line is drawn at the 50 per cent inhibition level.

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